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**14. ABSTRACT**

Prostate cancer is the most common tumor in men, being the second leading cause of cancer death after lung cancer. Telomerase, the enzyme that catalyzes the addition of telomere repeats (TTAGGG) to the ends of chromosomes, is almost universally required for the long-term cell proliferation that is necessary for metastasis. Inhibition of telomerase leads to critically short telomeres and tumor cell death. In the present study, the effects of a new telomerase antagonist, imetelstat sodium (GRN163L), were evaluated in a panel of prostate cancer cell lines. Administration of imetelstat resulted in the inhibition of telomerase activity in a dose-dependent fashion and progressive telomere shortening. Most prostate cancer therapies target the bulk tumor cells, but may leave intact a small population of tumor-initiating cells (also known as cancer stem cells). Using specific surface markers (CD44, integrin α2β1 and CD133), Hoechst 33342 dye exclusion, and holoclone formation, we isolated putative prostate tumor-initiating cells and demonstrate that that these cells have significant levels of telomerase activity. Most importantly, telomerase activity was inhibited by imetelstat in these stem-like prostate cancer cells. Because the average telomere length of prostate tumor-initiating cells was as short as or shorter than the average telomere size of the main population of cells we predict that imetelstat will be able to efficiently target the prostate tumor-initiating cells in addition to the bulk tumor cells.

**15. SUBJECT TERMS**

Telomerase, prostate, tumor-initiating cells, imetelstat

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## Introduction

Prostate adenocarcinoma is the most common type of cancer found in men, being second to lung cancer in terms of morbidity. While there are several therapeutic options (surgery, radiation) for the patients with localized disease, the patients with metastatic disease have only a few therapeutic options, all with limited efficacy. Therefore it is important to developed and implement new chemotherapeutic drugs for the treatment of prostate cancer. One of these novel therapeutic agents is imetelstat sodium (GRN163L), an oligonucleotide antagonist which targets the telomerase. Re-activation of telomerase, the enzyme responsible for maintenance of telomeres, is one of the hallmarks of cancer and more than 90% of prostate carcinomas possess this phenotype. In the absence of telomerase, cancer cells will progressively shorten their telomeres to a critical length and die. The majority of conventional chemotherapies are believed to leave intact small population of cells, known as tumor-initiating cells (or cancer stem cells). These cells are believed to be responsible for the subsequent relapse and metastasis of the disease and specific therapies which target these cells may achieve a more durable response. If tumor-initiating cells have telomerase activity, the imetelstat drug would target these cells in addition to the bulk tumor cells. In this study we are assessing the anti-metastatic effects of imetelstat by demonstrating that this drug targets the rare populations of tumor-initiating cells by inducing telomerase inhibition and telomere shortening.

## Body

The purpose of this research project was to assess the effects of a novel telomerase inhibitor drug, imetelstat, on prostate tumor-initiating cells. The initial working hypothesis was that by targeting the tumor-initiating cells we can reduce or eliminate the incidence of metastasis.

**Task 1. Determine the levels of telomerase activity in putative prostate tumor-initiating cells isolated from *in vitro* cultures and demonstrate inhibition of telomerase in these cells using a novel telomerase inhibitor, imetelstat (Months 1-9)**

Prior to the completion of this study it was completely unknown whether telomerase is active in prostate tumor-initiating cells. Moreover, the effects of imetelstat on prostate cancer cell lines were poorly understood. In order to address these questions, we analyzed the effects of imetelstat on several telomerase-positive prostate cell lines (DU145, PC3, C4-2 and LNCaP). All these cell lines have significant levels of telomerase activity, in contrast to normal prostate epithelial cells which are telomerase-negative (Figure 1). Most important, the Telomeric Repeat Amplification Protocol (TRAP) assay has shown that imetelstat treatment induced telomerase inhibition in all the cell lines analyzed (Figure 2). As expected, sustained telomerase inhibition with 2μM imetelstat led to telomere shortening as illustrated by the Terminal Restriction Fragment (TRF) using a telomere repeat probe (Figure 3). After prolonged imetelstat treatment, the telomeres became critically short and all the cell lines used in the experiments entered apoptosis and died (data not shown).

a. Isolation of prostate tumor-initiating cells from cancer cell lines (DU145, PC3, C4-2 and LNCaP) based on surface markers, Hoechst dye exclusion and formation of holoclones (Months 1-3)

There are several strategies employed to isolate prostate tumor-initiating cells, majority of these using established surface markers. In addition to these surface markers,

we isolated prostate tumor-initiating cells using Hoechst dye exclusion ("side population") and holoclones formation.

The first surface marker used for the isolation of prostate tumor-initiating cells in this study was CD44. CD44+ prostate cancer cells are more proliferative, clonogenic, tumorigenic and metastatic than the corresponding CD44- cells and possess traits of tumor stem/progenitor cells. Subsequently, it was found that $CD44^{hi}$/integrin $\alpha_2\beta_1^{hi}$ cells have higher tumorigenic potential than $CD44^{low}$/integrin $\alpha_2\beta_1^{low}$ cells, indicating that prostate cancer cells are organized in a hierarchy. Using the surface markers CD44 and integrin $\alpha_2$, we isolated a population of putative prostate tumor-initiating cells from DU145, an androgen-independent line. We sorted (FACS) the top 10% cells stained with each antibody, which translated to approximately 2% of the total population (Figure 4A).

The second surface marker used for the isolation of prostate tumor-initiating cells was CD133 (Prominin 1). Prostate cancer cells with the CD133+ phenotype show significant capacity of self-renewal as well as increased proliferation and differentiation potential. Using an antibody against CD133 (prominin-1), we detected a small population of CD133+ cells in the DU145 cell line (Figure 4B). All the cells lines analyzed show a small population of CD133+ cells (data not shown), but so far only DU145 CD133+ cells were shown to have tumor-initiating properties, therefore we used this cell line for the subsequent experiments.

The second strategy used to isolate prostate tumor-initiating cells employed the Hoechst 33342 dye exclusion assay, also known as the "side population" assay. The cells isolated from the side population are believed to harbor the tumor-initiating compartment. Using the Hoechst exclusion assay, we detected a small side population of C4-2 prostate cancer cells, which accounted for ~0.1% of the total population (Figure 6A). These cells were described in the literature as being more tumorigenic and drug resistant than the main population of cells, therefore providing an ideal model for testing the telomerase-inhibition therapy.

Another innovative strategy used to isolate prostate tumor-initiating cells is based on the hypothesis that only holoclones (tightly packed round colonies of cells with distinct morphology), are able to initiate tumor growth, and that these holoclones are maintained in carcinoma cell lines maintained in vitro. Holoclones contain self-renewing

tumor-initiating cells with high levels of CD44, integrin $\alpha_2\beta_1$ and β-catenin. We used very stringent criteria to isolate several DU145 holoclones (Figure 5) and expanded them briefly in culture, just long enough to harvest enough material for the subsequent assays.

For the PC3 cell line, we sorted both CD44-/+ and CD133-/+ cells in order to compare the telomerase activity in the positive and negative fractions for each marker. For the LNCaP cells we used a combination of CD44 and CD24 antibodies to identify a population of tumor-initiating cells with the CD44+/CD24- phenotype. The percentage of cells that stained positive for CD44 and negative for CD24 was relatively small, less than 1% (Figure 6B). A small population of CD133+ cells was also identified in the LNCaP cells. As mentioned before, the choice of cell line/isolation method was based on the data available in literature describing the tumorigenic potential of these populations of cells.

b-d. Perform TRAP analysis to verify telomerase activity in the prostate tumor-initiating cells and confirm inhibition of telomerase activity by imetelstat in these cells (Months 3-9)

After analyzing the effects of telomerase inhibition on the total population of cells (DU145, PC3, C4-2 and LNCaP), we proceeded to examine the telomerase activity in the prostate tumor-initiating cells, and investigate whether the telomerase inhibitor imetelstat is efficient in targeting these elusive cells. Equal numbers of tumor-initiating cells were collected for the untreated and imetelstat-treated samples and used for the TRAP assay. The DU145 $CD44^{hi}$/integrin $\alpha_2\beta_1{}^{hi}$ cells have telomerase levels similar to that of total population, and imetelstat inhibition of telomerase activity is equally efficient in this fraction (Figure 7A). DU145 CD133+ cells have high levels of telomerase activity, similar to the levels found in the total population of cells, and imetelstat is effective at inhibiting telomerase in these putative stem-like cells (Figure 7B). As illustrated in Figure 8, telomerase activity in DU145 holoclones was slightly lower than in the DU145 total population. The PC3 CD44+ and CD133+ have strong telomerase activity and no major differences were observed between the positive and negative fractions of cells (Figure 9A). Imetelstat was used to inhibit telomerase activity in all the isolated cell fractions. TRAP assays show that the SP cells isolated from the C4-2 cells have high

levels of telomerase activity, slightly higher than the main population of cells, and the enzyme's activity was inhibited efficiently by imetelstat (Figure 10A). The LNCaP CD44+/CD24- cells had high levels of telomerase activity, which was similar to the main population of LNCaP cells. Again, imetelstat was able to robustly inhibit telomerase activity in both fractions (Figure 10B). The CD133+ LNCaP cells have also strong telomerase activity which can be inhibited by imetelstat (Figure 9B).

Once we established that the prostate tumor-initiating cells have significant levels of telomerase activity, and that telomerase inhibitors can target efficiently these cells, we investigated the average telomere lengths of putative prostate tumor-initiating cell fraction. As seen in Figure 11, telomeres of prostate tumor-initiating cells are generally of similar average size with the main population of cells from which they were isolated. This predicts that telomere attrition should occur at equal rates in these cells. In order to verify this hypothesis, we isolated tumor-initiating cells from cell cultures treated with imetelstat for longer periods of time. As illustrated in Figure 11, the telomerase inhibitor was able to induce telomere shortening in these cells, and it appears that the rate of telomere shortening in these cells is similar to that found in the main population of cells.

When we compared the FACS profile of cells treated for different periods of time with imetelstat, we observed that the proportion of DU145 $CD44^{hi}$/integrin $\alpha_2\beta_1^{hi}$ cells present in the total population decreased proportional with the length of imetelstat treatment (Figure 12A). Moreover, after ~100 days of treatment we were unable to detect any holoclone formation when the cells were plated at low density (Figure 12B) which correlates with the clonogenic spheroid formation assays which showed that prolonged treatment with imetelstat leads to decreased spheroid formation ability in all of the cell lines analyzed (Figure 12C). This was the first study suggesting that long-term treatment with imetelstat may lead to the elimination of tumor-initiating cells. This phenomenon was confirmed by subsequent studies performed by other researchers in multiple myeloma and breast cancer. Whether this is the direct result of telomerase inhibition and telomere shortening remains unclear and future experiments will be aimed at answering this important question.

Because the tumor-initiating cells are present in very small numbers in the cell lines analyzed, we examined the effects of culture conditions on sorted populations of

cells in an attempt to enrich the population for tumor-initiating cells. The CD133+ cells isolated from DU145 cells were placed back in culture, using both serum-supplemented media and adherent conditions or chemically defined media and attachment-independent conditions (spheroids). Regardless of the media and culture conditions used, the percentage of cells expressing CD133 remained very low (less than 1%), without any apparent enrichment over time in culture (Table 1). Next, we examined by immunofluorescence imaging the signature of DU145 prostate cancer cells grown at clonal density in attachment-independent conditions (spheroids). The attachment-independent conditions exert even more strain on the cells, and because spheroid formation was used extensively to enrich for stem cells, the clonogenic spheroid formation assay probably identifies the population of cells that have the highest tumorigenic potential. We specifically focused on common tumor-initiating cells markers such as CD44 and CD133. CD44 is present at high levels in the majority of DU145 cells, regardless of culture conditions (monolayer or spheroids). The CD133+ cells were also clearly identified in the spheroids, but as the spheroids grew in size, the CD133+ population did not proliferate at the same rate as the CD44 cells within the spheroids (Figure 13). While these spheroids were grown in serum-supplemented media (that usually promotes differentiation), the use of serum-free defined media also did not enrich the CD133+ population. This observation suggests that the culture conditions we used do not allow the enrichment of this rare population of cells.

**Task 2 and 3. Isolate putative prostate tumor-initiating cells and examine the role of imetelstat on telomerase inhibition and metastasis in orthotopic mice xenografts (Months 10-24):**

The luciferase prostate cancer cell lines DU145-luc, PC3-luc, C4-2-luc and LNCaP-luc were established using a lentivirus encoding the luciferase gene driven by an ubiquitin promoter. After infection and subsequent selection with G418 for two weeks, high levels of luciferase expression were detected in all the cell lines. In addition, we generated cell lines expressing GFP alone and in combination with luciferase.

The attempts to generate a reliable metastatic model using the luciferase-expressing cell lines engineered previously were unsuccessful. The incidence of metastasis varied greatly amongst the cell lines, and within the cell lines amongst animals, regardless of the inoculation site (data not shown).

The epithelial-to-mesenchymal transition (EMT) is a highly conserved cellular process that allows the polarized and generally immotile epithelial cells to convert to motile mesenchymal cells. This important process was initially recognized during several critical stages of embryonic development and has recently been implicated in promoting cancer invasion and metastasis. A decrease in the expression of E-cadherin and an increase in the expression of vimentin are two currently accepted biochemical characteristics associated with EMT. In prostate cancer, decreased E-cadherin expression has been correlated with various indices of prostate cancer progression, including grade of the cancer, local invasiveness, dissemination into blood, and tumor relapse after radiotherapy. Most important, recent reports suggested that the EMT process generates cells with characteristics of tumor-initiating cells. This concept led us to the idea of developing a prostate EMT model in order to improve the incidence of metastasis in the mouse model.

The PZ-HPV-7 cell line was derived from histological normal prostate epithelium immortalized by Human Papilloma Virus Type 18 (HPV-18) DNA. PZ-HPV-7 cells are generally considered as non-tumorigenic in subcutaneous xenograft animal models. Nevertheless, we have established a new PZ-HPV-7T line from the parental PZ-HPV-7 cells, which is highly tumorigenic in nude mice and has increased expression of EMT markers, suggesting the progression to a more aggressive phenotype.

The PZ-HPV-7 cells are non-tumorigenic in nude mice (Table 2) when implanted by subcutaneous injection (0/2) or subrenal grafting (0/8). However, when PZ-HPV-7 cells were cultured as spheroids in Matrigel® then implanted under the renal capsule of nude mice, one mouse (1/5) developed a palpable xenograft tumor (Figure 14A and Table2). The tumor was excised, placed back into cell culture and the cell line generated from this xenograft explant was designated PZ-HPV-7T. The PZ-HPV-7T cells appeared to be more tumorigenic and metastatic than the parental PZ-HPV-7 cells (Table 2). Mice inoculated with PZ-HPV-7T cells under the renal capsule exhibited multiple sites of

metastases such as lymph nodes and lungs (Figure 14A). In addition to its capacity to form tumors in the subrenal capsule of nude mice, PZ-HPV-7T cells were able to generate subcutaneous tumors in NOD/SCID mice (Table2). In two-dimensional cultures on plastic dishes, PZ-HPV-7 cells have typical epithelial morphology, but the PZ-HPV-7T cells possess a more mesenchymal-like appearance (Figure 14B).

PZ-HPV-7 cells are hypertriploid with the chromosome number ranging from 78 to 85, with deletion of chromosomes 3p, 15, 21, and 22, as well as gain of chromosomes 3q, 5, 5p, 7, 7p, 9q, 11, 16p, 17, 18, 19, 20, and 3 to 7 marker chromosomes (Figure 15A). PZ-HPV-7T cells are hyperdiploid with the chromosome number ranging from 47 to 57. Deletion of chromosomes 4, 7q, 16q, 18, and 20 as well as gain of chromosomes 1, 5p, 6p, 8, 9q, 10q, 11q, and 17 can be observed (Figure 15B). Structural anomalies including an additional isochromosome 5p and a 7q deletion from 7q11.2 to the 7qter region were present in both cells, supporting the common origin of these two cell lines.

The expression levels of established EMT markers such as ZEB1 and ZEB2 were significantly elevated in PZ-HPV-7T cells, regardless of the culture conditions (Figure 16A). The expression of Snail and FOXC2 mRNA, two other key players in the EMT process, were also significantly elevated in PZ-HPV-7T cells in both media. Similarly, vimentin, N-cadherin and fibronectin, bona fide mesenchymal markers, were elevated in the PZ-HPV-7T cells (Figure 16B) in both culture media. In contrast, E-cadherin, an epithelial marker, decreased significantly in PZ-HPV-7T cells compared with PZ-HPV-7 cells (Figure 16B). Interestingly, decreased expression of DAB2IP protein, a critical factor in preventing EMT, was also detected in PZ-HPV-7T cells but not the PZ-HPV-7 cells. This indicates that PZ-HPV-7T cells have undergone distinct EMT that correlates with its tumorigenicity and metastatic potential in vivo.

The PZ-HPV-7T cell line provides a reliable metastatic model which will facilitate the study of imetelstat as an antimetastatic agent in mouse xenografts.

## Key research accomplishments

1. The telomerase inhibitor imetelstat induced telomerase inhibition and telomere shortening in four prostate cancer cell lines (DU145, PC3, C4-2 and LNCaP). When the telomeres became critically short, the cells ceased to proliferate and died.
2. Putative prostate tumor-initiating cells have significant levels of telomerase activity. This is a new discovery and suggests that tumor-initiating cells are not quiescent, but actively dividing in the main population. Moreover, because these cells use telomerase as a telomere maintenance mechanism, we identified a new therapeutic target and have initiated experiments to test a novel telomerase inhibitor, imetelstat that may prevent relapse and metastasis.
3. The prostate tumor-initiating cells have relatively short telomeres, similar in size to the telomeres of the bulk population of cells. This suggests that these cells (in addition to the bulk tumor cells) will be targeted first by telomerase inhibitor, before any negative effects will occur on normal stem cells (which have relatively long telomeres).
4. The telomerase inhibitor imetelstat can target efficiently the tumor-initiating cells. Imetelstat inhibited telomerase activity very efficiently in all the prostate tumor-initiating cell fractions isolated in this study. Moreover, prolonged telomerase inhibition by imetelstat leads to telomere shortening in the prostate tumor-initiating cells at a rate which appears similar to that observed in the main population of cancer cells.
5. Long-term telomerase inhibition reduces the number of tumor-initiating cells and leads to a decreased capacity of self-renewal in prostate cancer cell lines. This phenomenon is mainly the result of telomere shortening in the tumor-initiating compartment but we cannot reject the possibility that telomerase inhibitors (such as imetelstat) may have additional effects on the cancer cells, independent of telomere shortening. Nevertheless, this discovery has significant implications for the treatment of localized and metastatic disease.

6. The culture conditions used to propagate the prostate cancer cell lines used in this study do not promote enrichment for tumor-initiating cells. When CD133+ cells were sorted from the main population of cells and placed back in culture using two different media (with and without serum) in monolayer and suspension spheroids, the percentage of CD133 cells returned to the values identified in the initial population in a short amount of time.
7. We have developed a novel EMT model using an immortalized normal prostate epithelial cell line and generated a new prostate cancer cell line, PZ-HPV-7T, which may represent an excellent system to study mechanisms associated with prostate cancer progression and metastasis. This model is ideal for mouse xenograft studies with imetelstat.

## Reportable outcomes

### Publications

Marian CO, Yang L, Zhou Y, Pong RC, Gore C, Wright WE, Shay JW, Kabbani W, Hsieh JT and GV Raj (2011). Evidence of epithelial to mesenchymal transition associated with increased tumorigenic potential in an immortalized normal prostate epithelial cell line. Prostate 71:626-636.

Marian CO, Wright WE and Shay JW. (2010) The effects of telomerase inhibition on prostate tumor-initiating cells. International Journal of Cancer 127: 321-331.

Marian CO and Shay JW. (2009) Prostate tumor-initiating cells: A new target for telomerase inhibition therapy? Biochimica et Biophysica Acta 1792: 289-96.

### Abstracts

Marian CO, Wright WE and Shay JW. (2009) The effects of telomerase inhibition on prostate tumor-initiating cells. 100$^{th}$ AACR Meeting Denver, Colorado April 18-22.

## Conclusion

We established the presence of active telomerase in putative prostate tumor-initiating cells isolated from prostate cell lines and we have shown that the telomerase inhibitor imetelstat is able to inhibit the enzymatic activity in these cells with the same efficiency as in the main population. Telomerase inhibition induced progressive telomere shortening in tumor-initiating cells at the same rate found in the cell line from which they were derived. After telomeres become critically short, cells will enter apoptosis and die. Most important, prolonged treatment with imetelstat leads to a decrease in the number of tumor-initiating cells, opening new avenues of research. Thus, imetelstat may be a valuable therapy for the treatment of prostate cancer. Its unique mode of action indicates that it may target the elusive putative tumor-initiating cells that are usually resistant to conventional therapies.

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## Supporting data

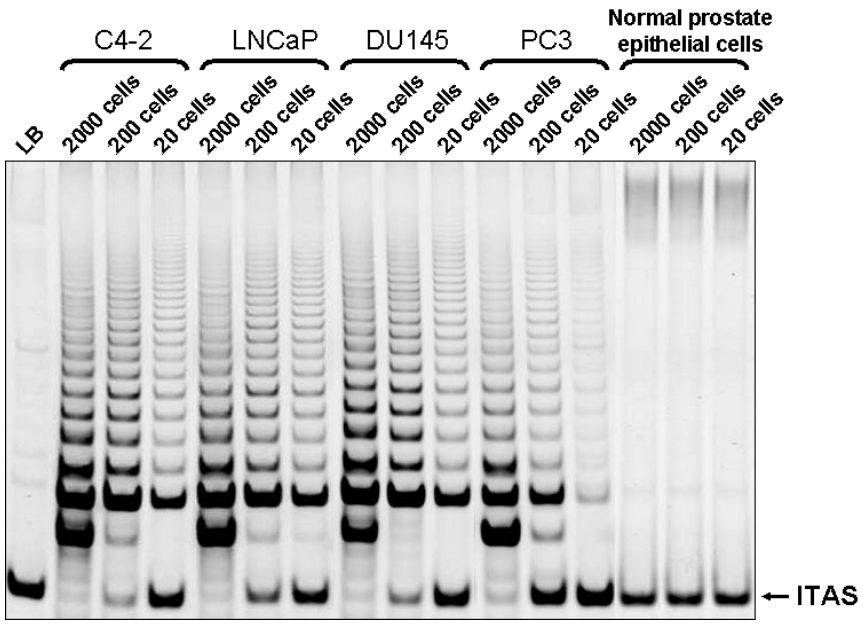


**Figure 1**. **Prostate cancer cell lines have telomerase activity**. Telomere Repeat Amplification Protocol (TRAP) on cell lysates from different prostate cell lines. Normal primary prostate epithelial cells are telomerase negative. The internal amplification standard used for quantifying telomerase activity levels is indicated by an arrow.

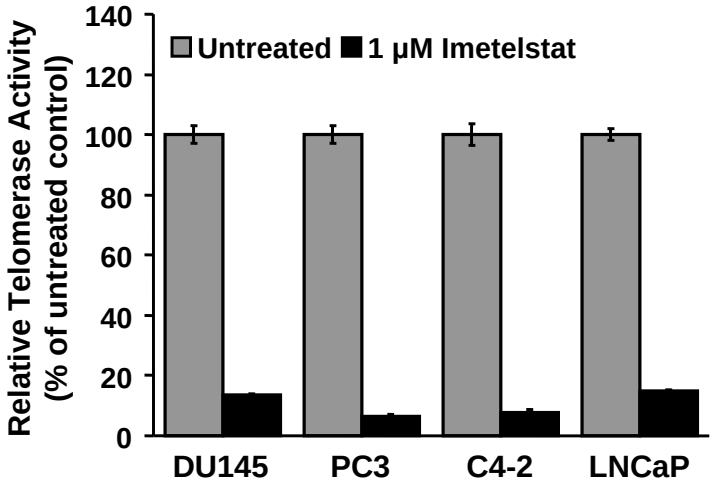


**Figure 2. Prostate cell lines have high levels of telomerase activity which can be inhibited by imetelstat.** Quantification of the TRAP signal presented as a ratio between the intensity of the telomerase ladder signal versus the intensity of internal amplification standard (ITAS) band. 1 μM imetelstat inhibits telomerase activity efficiently in all the cell lines analyzed. Relative telomerase activity was normalized to the untreated control cells. Lysate equivalent to the same number of cells was used for TRAP with all the cell lines.

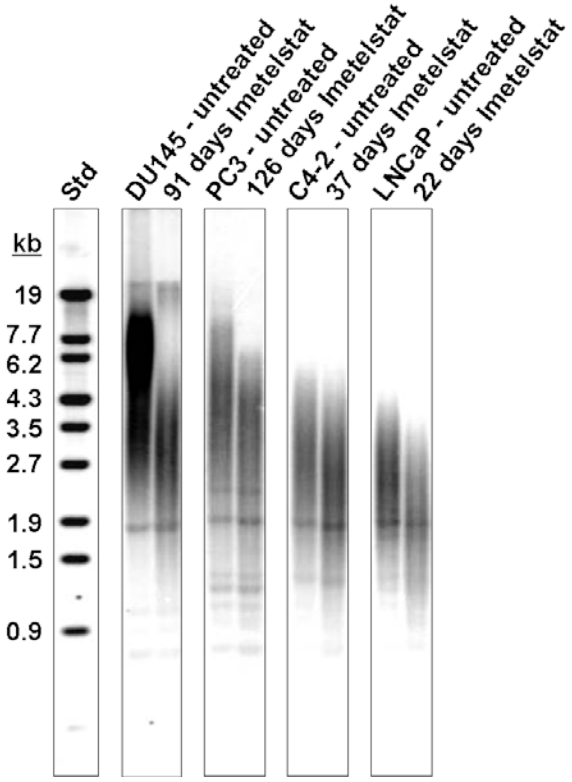


**Figure 3. Continuous inhibition of telomerase by imetelstat leads to telomere shortening.** Terminal restriction fragment (TRF) shows that sustained telomerase inhibition with 2 μM imetelstat leads to telomere shortening.

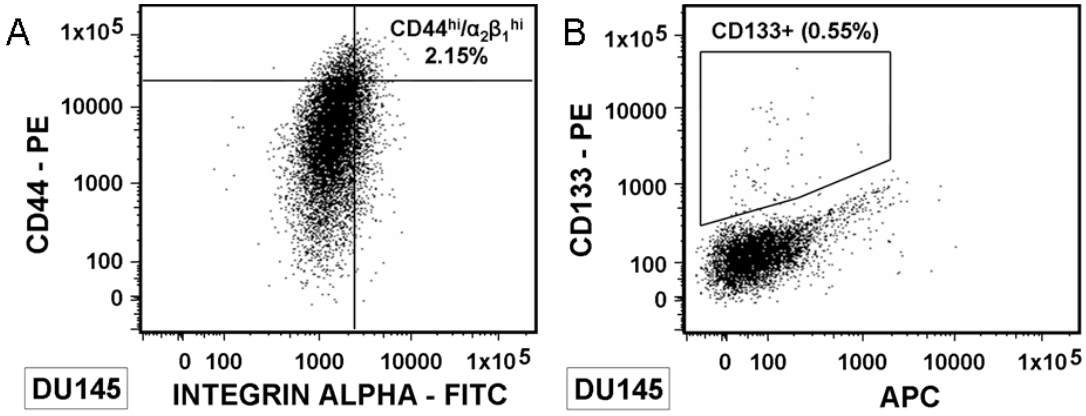


**Figure 4. Isolation of DU145 prostate tumor-initiating cells using surface markers. A.** DU145 cells with the $CD44^{hi}$/integrin $\alpha_2\beta_1^{hi}$ phenotype were isolated using fluorescence-activated cell sorting (FACS). **B.** DU145 cells contain a small population of CD133+ tumor-initiating cells. The CD44 and integrin-specific antibodies were purchased from BD Biosciences and the CD133 antibody from Miltenyi Biotec.

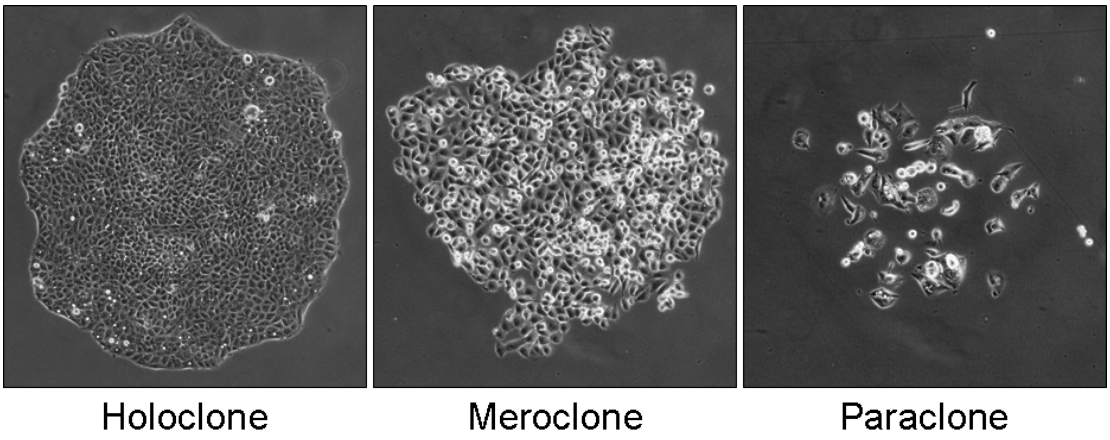


**Figure 5. Isolation of DU145 holoclones.** Characteristic morphology of DU145 clones; holoclones can be easily distinguished from paraclones.

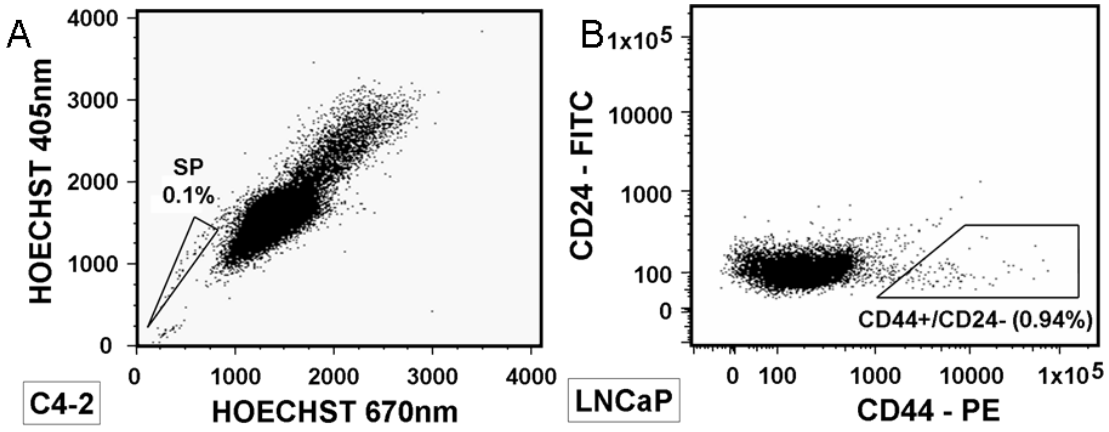


**Figure 6. Isolation of the side population (SP) and CD44+/CD24- cells. A.** The C4-2 side population was isolated based on Hoechst 33342 dye exclusion. **B.** LNCaP cells possess a small population of CD44/CD24- cells as illustrated by FACS.

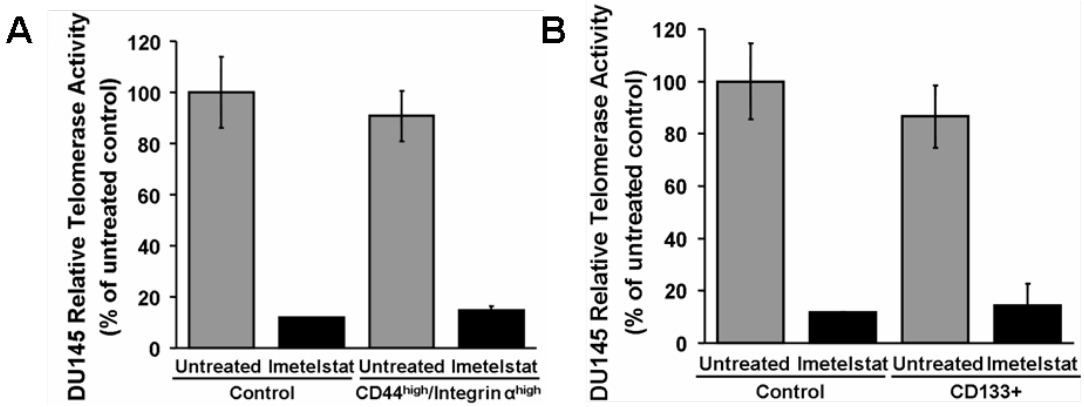


**Figure 7. Imetelstat acts efficiently on DU145 prostate tumor-initiating cells isolated using surface markers. A.** Relative telomerase activity in the untreated and imetelstat-treated cells for total and $CD44^{hi}$/integrin $\alpha_2\beta_1^{hi}$ cells. **B.** Relative telomerase activity in DU145 CD133+ cells compared to the total population of cells. Imetelstat inhibits telomerase activity in the CD133+ cell population. The cells were treated with 1μM imetelstat before sorting and analysis.

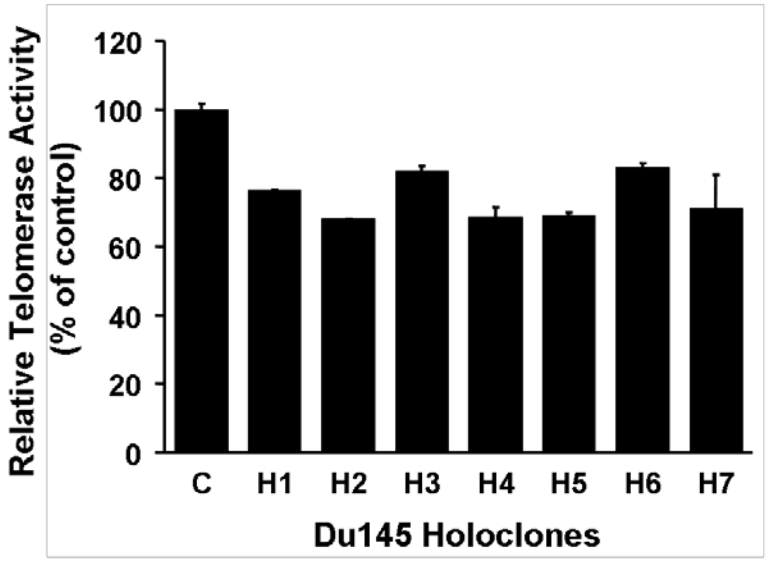


**Figure 8. D U145 holoclones have significant levels of telomerase activity.** Relative telomerase activity in several DU145 holoclones measured using the TRAP assay.

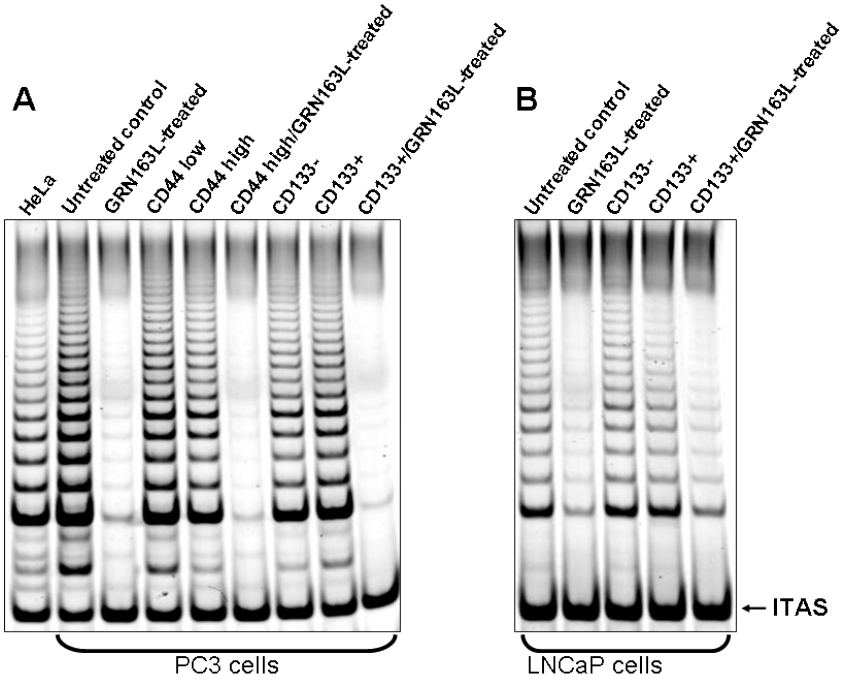


**Figure 9. Putative prostate tumor-initiating cells have telomerase activity which can be inhibited by telomerase inhibitors (imetelstat)** A. TRAP assay on CD44 and CD133 populations of cells isolated from PC3 cells. B. TRAP assay on isolated CD133 LNCaP cells. The cells were pre-treated for 72 hours with the telomerase inhibitor drug (2 μM) before sorting. HeLa cells were used as positive controls. The internal amplification standard is indicated by an arrow.

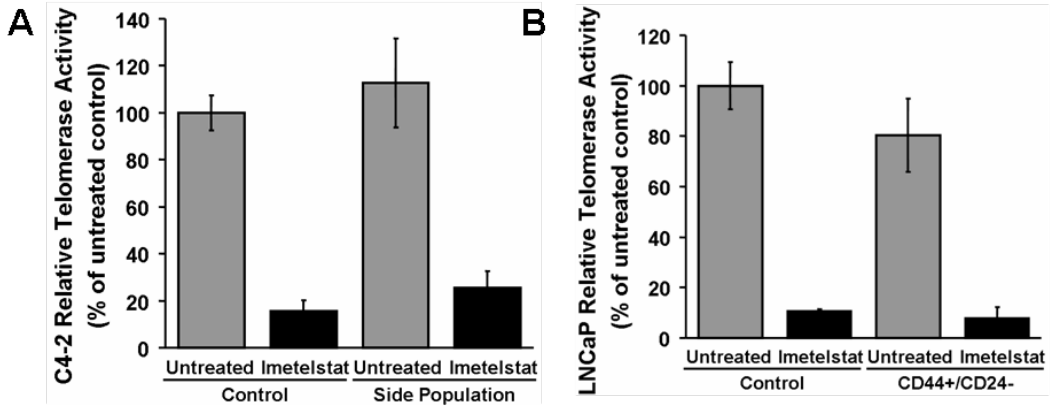


**Figure 10. Side population (SP) and CD44+/CD24- cells are telomerase positive and sensitive to imetelstat. A.** C4-2 SP cells have similar telomerase activity to the main population of cells and imetelstat-mediated telomerase inhibition is equally efficient in both fractions. **B.** Relative telomerase activity in the untreated and imetelstat-treated cells for total and CD44+/CD24- cells. The cells were treated with 1μM imetelstat before sorting and analysis.

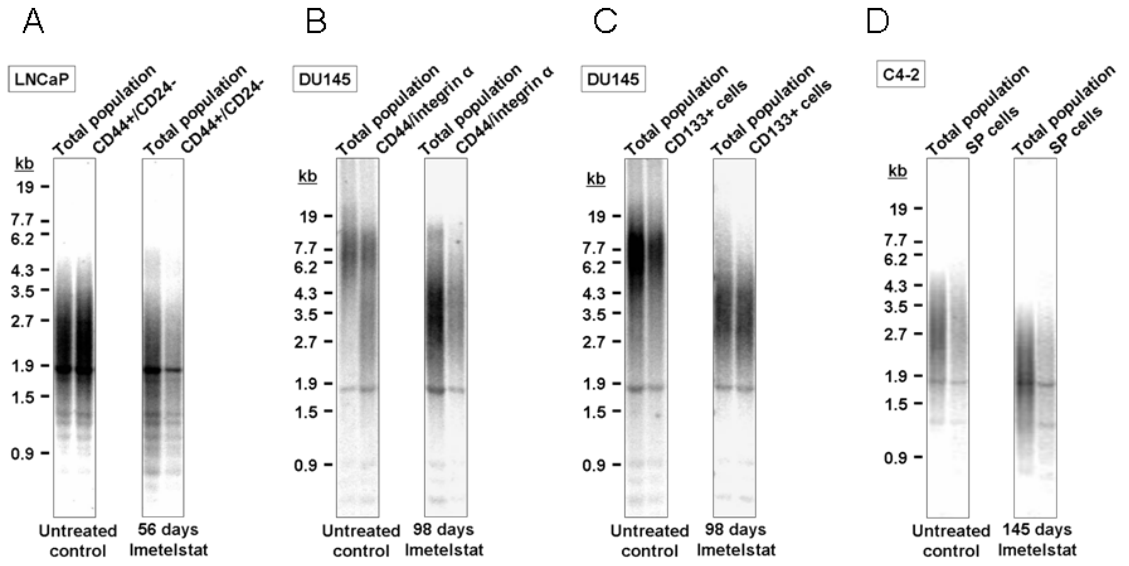


**Figure 11. Telomere lengths in prostate tumor-initiating cells compared with the telomeres of untreated and imetelstat-treated cells. A.** LNCaP cells were treated with 2μM imetelstat for 56 days then the CD44+/CD24- cell fraction was isolated and TRF assay performed on the extracted genomic DNA. **B.** DU145 cells were treated with imetelstat for 98 days then the $CD44^{hi}$/integrin $\alpha_2\beta_1{}^{hi}$ population was isolated by FACS and TRF performed on total genomic DNA extracted from the cells. **C.** TRF on total genomic DNA extracted from DU145 CD133+ cells treated with imetelstat for 98 days was compared with the telomeres signal obtained from the untreated total population of cells. **D.** The C4-2 side population is sensitive to telomere erosion effects of imetelstat similarly to the main population of cells.

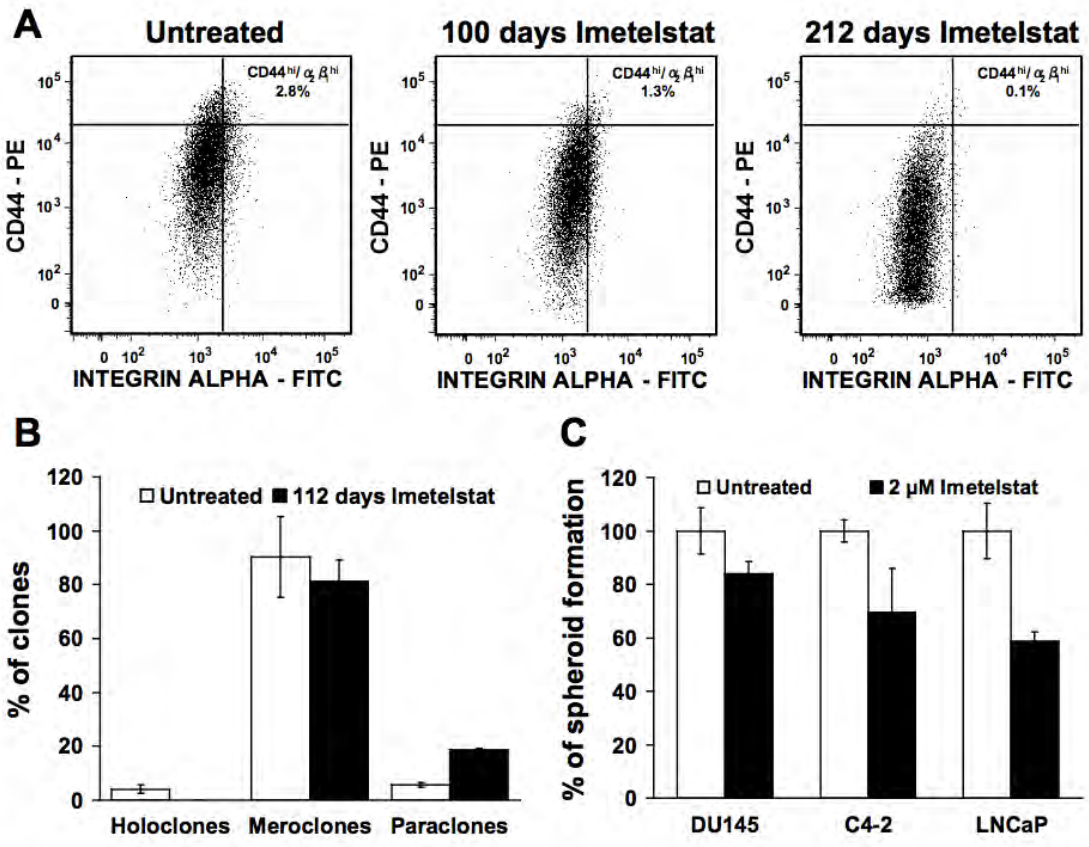


**Figure 12. Sustained telomerase inhibition by imetelstat in DU145 cells might lead to a decrease in the number of tumor-initiating cells. A.** FACS analysis of imetelstat-treated DU145 cells over long periods of time indicates a decrease in the $CD44^{hi}$/integrin $\alpha_2\beta_1^{hi}$ tumor-initiating population of cells. **B.** After prolonged treatment with imetelstat, the capacity of DU145 cells to generate holoclones was completely abolished while the number of paraclones increased. **C.** Prostate cancer cell lines treated with 2μM imetelstat (56 days for LNCaP and C4-2; 98 days for DU145) show decreased capacity of self-renewal as indicated by the clonogenic spheroid formation assay.

**Table 1. The percentage of DU145 CD133+ cells is maintained at low levels in culture after initial FACS isolation in both monolayer and spheroid cultures**

| Percentage of DU145 CD133+ cells | | | |
|---|---|---|---|
| **Days in culture** | **14** | **30** | **33** |
| **Monolayer*** | 0.19 | 0.19 | 0.18 |
| **Spheroids**** | 0.21 | 0.19 | 0.21 |
| *** The sorted CD 133+ cells were cultured in DMEM/199 media with 10% FBS** | | | |
| **** The sorted CD133+ cells were cultured as spheroids in DMEM/F12 media** | | | |

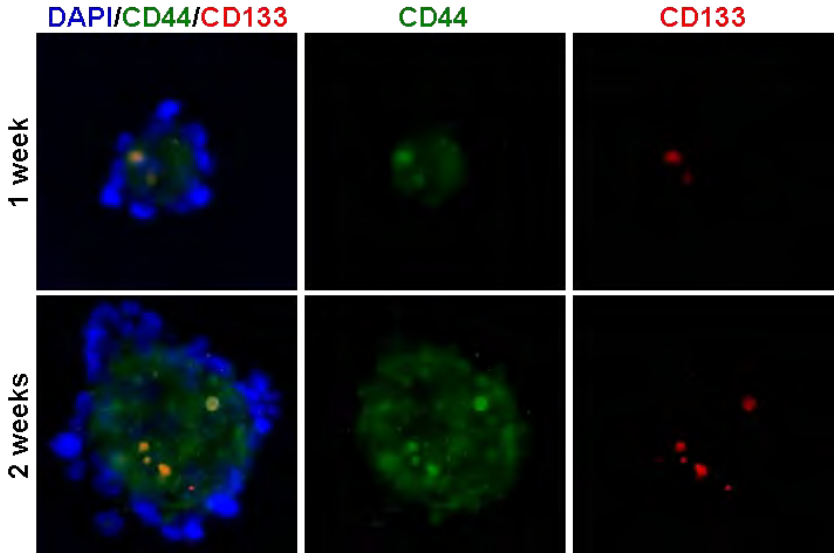


**Figure 13. Clonogenic prostate cancer spheroids contain CD133+ cells** DU145 prostate cancer cells were plated at low density in attachment-independent conditions and immunostaining was performed one and two weeks after plating. The primary conjugated antibodies CD44-FITC (BD Biosciences) and CD133-PE (Miltenyi Biotech) were used (1:100) and the spheroids were counterstained with DAPI. Magnification is 20X.

**Table 2. Xenograft tumor formation in immunocompromised mice**

| CELL TYPE/GROWTH CONDITIONS | INOCULATION SITE | NO. OF MICE | % TUMORS |
|---|---|---|---|
| PZ-HPV-7/monolayer | Subrenal | 8 | 0% (0/8) |
| PZ-HPV-7/spheroids | Subrenal | 5 | 20% (1/5) |
| PZ-HPV-7/monolayer | Subcutaneous | 2 | 0% (0/2) |
| PZ-HPV-7T/monolayer | Subrenal | 4 | 100% (4/4) |
| PZ-HPV-7T/spheroids | Subrenal | 4 | 75% (3/4) |
| PZ-HPV-7T/monolayer | Subcutaneous | 2 | 100% (2/2) |

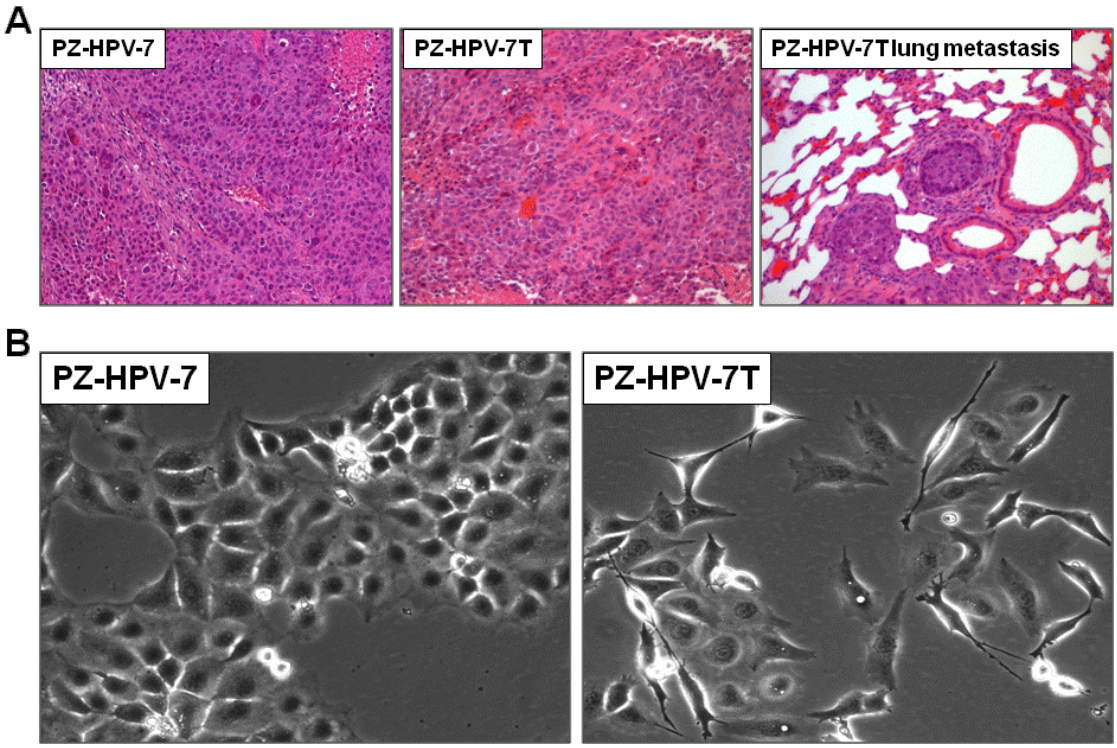


**Figure 14. The morphology of PZ-HPV-7 and PZ-HPV-7T cells and the histology of tumors in mouse xenografts A)** H&E staining of the mouse subrenal xenograft tumors generated from PZ-HPV-7 and PZ-HPV-7T cells. The PZ-HPV-7T xenografts show evidence of lung metastasis. **B)** Morphology of the PZ-HPV-7 and PZ-HPV-7T cells growing on a on a plastic substrate.

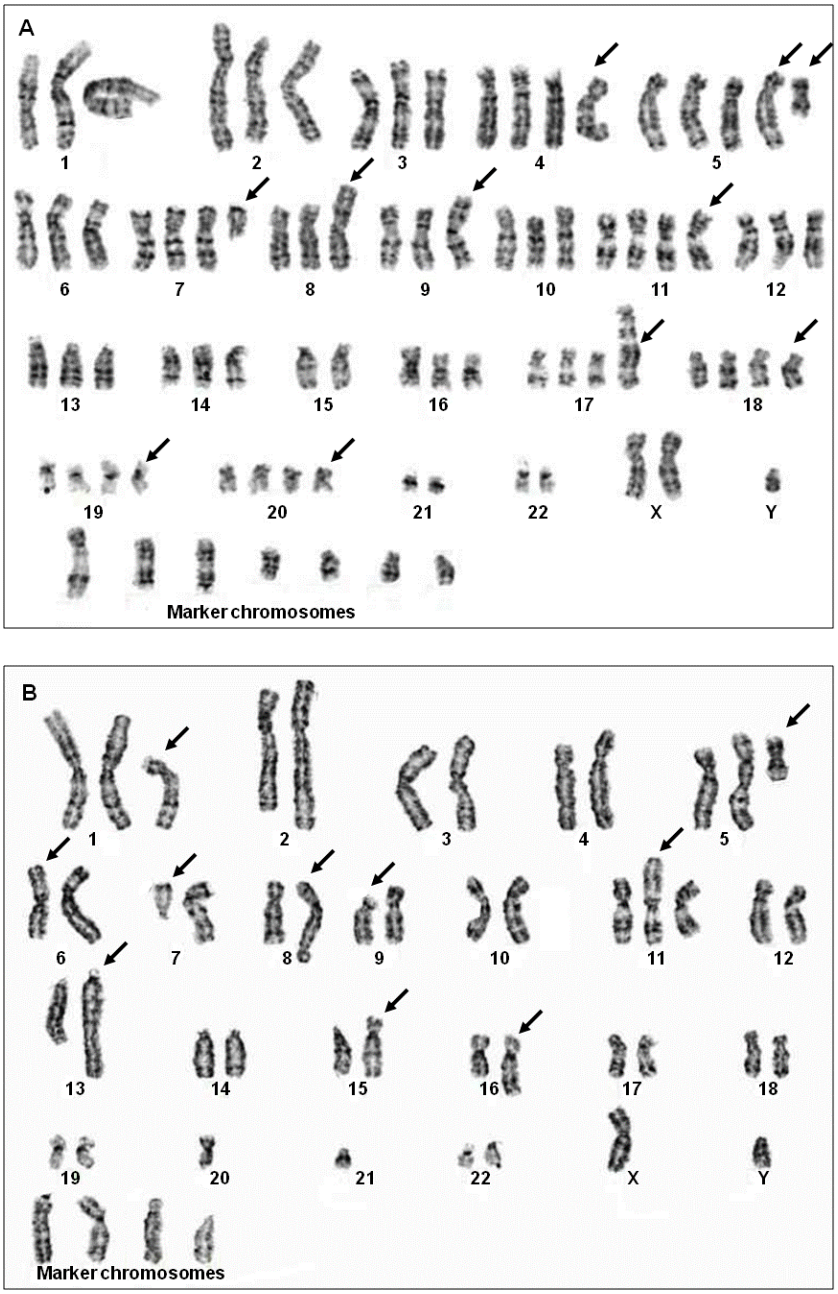


**Figure 15. Representative karyotypes for PZ-HPV-7 and PZ-HPV-7cells. A)** Late passage PZ-HPV-7 cells were hypertriploid with the following karyotype: 82<3n>, XXY, +4, +5, +i(5)(p10), +del(7)(q11.2), der(8)t(8;10)(p11.2;q11.2), i(9)(q10), +11, -15, +der(17)t(5;17)(q13;q25), +18, +19, +20, -21, -22, +7mar; **B)** PZ-HPV-7T cells presented a hyperdiploid karyotype with: 51<2n>, XY, +t(1;11)(p22;p11.2), +i(5)(p10), der(6)add(6)(p23)del(6)(q23), del(7)(q11.2), add(8)(q24), del(9)(p13), add(13)(q34), add(15)(p12), add(16)(q22), -20, -21, +4mar.

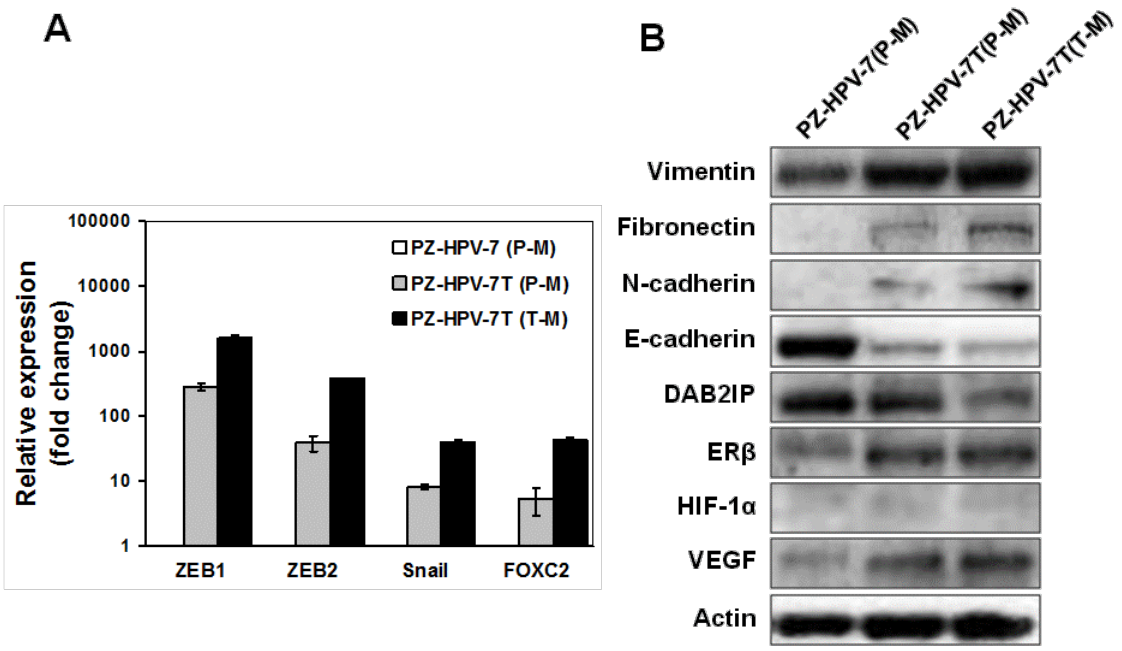


**Figure 16. Characterization of EMT changes in PZ-HPV-7 and PZ-HPV-7T cells. A)** Steady-state mRNA levels of ZEB1, ZEB2, Snail and FOXC2 in PZ-HPV-7 and PZ-HPV-7T cells were determined by qRT-PCR. Asterisk represented statistical significant between PZ-HPV-7T and PZ-HPV-7 ($p<0.05$). **B)** Steady-state protein levels of vimentin, fibronectin, N-cadherin, E-cadherin and DAB2IP in PZ-HPV-7 and PZ-HPV-7T were analyzed by western blots. Actin was used as loading control. The cells were grown in Matrigel® as spheroids in two different media (P-M: PrEGM medium, T-M: T-medium).

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Review

# Prostate tumor-initiating cells: A new target for telomerase inhibition therapy?

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ABSTRACT

Conventional therapies for prostate cancer, especially in its androgen-independent form, may result in the survival of small populations of resistant cells with tumor-initiating potential. These "cancer stem cells" are believed to be responsible for cancer relapse, and therapeutic strategies targeting these cells are of great importance. Telomerase is a ribonucleoprotein enzyme responsible for telomere elongation and is activated in the majority of malignancies, including prostate cancer, but is absent in most normal cells. Putative tumor-initiating cells have significant levels of telomerase, indicating that they are an excellent target for telomerase inhibition therapy. In this review, we present some evidence for the hypothesis that conventional therapies (standard chemotherapy and/or radiation therapy) in combination with telomerase inhibitors may result in effective and more durable responses.



## 1. Introduction

Prostate cancer is the second most common malignancy found in men and is responsible for the highest rate of morbidity after lung cancer [1]. In most cases, localized prostate disease can be treated efficiently using surgery and androgen ablation therapy. However, the outcome for patients with metastatic disease remains poor [2]. Considering the advanced age for the majority of patients, the chemotherapy regimens have done little to improve median survival, and the lethality of the disease in patients with metastatic castrate-resistant disease remains high [3]. The Gleason classification of prostate tumors remains the best predictor for disease outcome, but more recently new molecular diagnostic techniques such as identification of TMPRSS2:ERG fusion transcripts [4,5], Glutathione-S-transferase P1 (GSTP1) gene promoter hypermethylation [6,7] and DD3 expression [8] can assist in early detection, prognosis, and monitoring of prostate cancer. In addition to diagnostics, current research in the prostate cancer field is focused on the establishment of new targeted therapies for the patients with metastatic disease. It is generally believed that cancer relapse in patients may be due to a small population of cells within the tumor mass which are resistant to conventional therapies.

## 2. The cancer stem cell hypothesis

The cancer stem cell hypothesis was described more than 150 years ago [9], but the modern revival of this concept arrived with the studies performed in leukemia, where it was shown that a single cell with the CD34+/CD38− phenotype had the capacity of inducing the disease in NOD-SCID mice [10]. More recently, cancer stem cells have been isolated from solid tumors, first in breast cancer, then in neurological malignancies [11,12]. The term "cancer stem cells" is still very controversial. Nevertheless, the general consensus is that these cells must have potent tumor initiation, self-renewal and differentiation capacity [13]. The tumor initiation aspect refers to the capacity of these cells to form tumors in immunocompromised mice using very small numbers of cells. Self-renewal capacity is tested by serial transplantation experiments, where re-isolated cancer stem cells can be transplanted in secondary and tertiary recipients. The differentiation ability of these cells does not refer to multilineage differentiation but rather to the capacity of the resulting tumors to be a phenocopy of the original tumor. An important characteristic of cancer stem cells is their ability to survive various therapies by activating anti-apoptotic pathways, increasing activity of membrane transporters and high DNA repair capacity [14,15]. It is important to point out that the definition of cancer stem cells does not imply the cell type from which these cells originated. This is the reason why for the purpose of this review we are going to use the term tumor-initiating cells. While the origin of tumor-initiating cells is highly debated, this review will focus on the intrinsic properties of these cells in prostate cancer, specifically on telomerase as both a biomarker and therapeutic target for this type of malignancy. The study of these populations of cells is very important, not only for the basic understanding of malignant transformation and pathogenesis, but also as a way to investigate and implement new therapies.

## 3. Isolation of prostate tumor-initiating cells

To some extent, the amount of knowledge about prostate tumor-initiating cells is still limited compared to that reported in other

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cancers types. This is due, in part, to the small amounts of primary tumors samples available for investigation, and the difficulty in distinguishing between normal and malignant prostate cells based on surface markers alone. Because prostate tumor-initiating cells are present in very low numbers within a primary tumor (usually less than 1%), the use of cancer cell lines provides an efficient alternative to clinical samples. The caveat is that one needs to validate any scientific knowledge derived from prostate cell lines with studies in primary tumor counterparts. Cell lines are usually grown in culture medium supplemented with serum and high $Ca^{2+}$, conditions that generally permit growth but also encourage differentiation. Some researchers are strong advocates of xenograft propagation of human tumors, but the mouse environment is very different from the human prostate stromal niche, especially when using subcutaneous or renal capsule inoculations, and some amount of differentiation is unavoidable. An alternative is the prostate orthotopic xenograft, but these are difficult to establish, with high rates of mortality. The combinatorial use of primary samples, xenografts and cell lines will likely provide the tools for the most rigorous scientific investigations.

There are several strategies to isolate prostate tumor-initiating cells. The most popular strategy employs the use of surface markers that share the same immunological profile with normal prostate stem cells. One of these surface markers is CD44, an adhesion molecule with multiple functions that appears to be important in tumor dissemination and metastasis [16–18]. One research group reported an in-depth study using $CD44^{high}$ cells isolated from various prostate cell lines [19]. These putative tumor-initiating cells were more proliferative, clonogenic, tumorigenic, and metastatic than the $CD44^{low}$ cells. The CD44 cells also show properties of progenitor cells, such as BrdU label retention and expression of several "stemness" factors, such as Oct-3/4, BMI, β-catenin, and SMO. Moreover, while these cells were AR−, they had the capacity to differentiate into AR+ cells. The authors recognized that the $CD44^{high}$ population of cells was still very heterogeneous and tried to further purify the tumor-initiating component using additional surface markers. In a subsequent study, it was shown that $CD44^{high}/\alpha_2\beta_1$ $integrin^{high}$ populations were more tumorigenic than $CD44^{low}/\alpha_2\beta_1$ $integrin^{low}$ populations when injected in immunocompromised mice and the authors proposed a tumorigenic hierarchy of prostate cancer cells based on the expression of these two markers [20].

Based on the similarities between mouse prostate and breast stem-like cells, another study sought to determine if a population of CD44+/CD24− cells identified tumor-initiating cells in the LNCaP prostate cancer cell line [21]. These cells were present at a very low level in the population (0.04%), and show increased clonogenic and differentiation capacity. Importantly, very low numbers of CD44+/CD24− cells were capable of forming tumors in NOD/SCID mice. These cells were also able to grow as spheroids in attachment-independent conditions and possessed an invasive gene signature.

Another important stem cell marker is prominin-1 (CD133), a pentaspan membrane protein with unclear function [22]. Collins et al. used a $CD44/\alpha_2\beta_1$ $integrin^{high}$/CD133+ phenotype to isolate tumor-initiating cells from primary prostate biopsies [23]. The cells isolated with these markers have a high clonogenic and proliferative capacity, are highly invasive through matrigel and capable of differentiation.

The percent age of CD133+ cells is low after the initial purification from primary tumor samples. Using the prostate cancer cell line DU145 we also find low numbers of CD133+ cells (Table 1). CD133+ cells isolated from primary tumors or DU145 cells can be placed back in culture, using both serum-supplemented media and adherent conditions or chemically defined media and attachment-independent conditions (spheroids). Regardless of the media and culture conditions used, the percentage of cells expressing CD133 remains very low (less than 1%), without any apparent enrichment over time in culture. This indicates that the culture conditions commonly employed in vitro for the propagation of prostate tumor stem cells do not allow the enrichment of this rare population of cells. This is in stark contrast with brain tumor stem cells, where some degree of positive enrichment is possible when isolated CD133+ cells are placed in culture [12]. Importantly, the experiments performed in DU145 cells indicate that the biology of CD133+ cells in primary tumor samples and cancer cell lines might be similar, and if true, the prostate cancer cell lines can be used as a valuable source of research material.

**Table 1**
The percentage of DU145 CD133+ cells is maintained at low levels in culture after initial FACS isolation in both monolayer and spheroid cultures

| Percentage of DU145 CD133+ cells | | | |
|---|---|---|---|
| Days in culture | 14 | 30 | 33 |
| Monolayer[a] | 0.19 | 0.19 | 0.18 |
| Spheroids[b] | 0.21 | 0.19 | 0.21 |

[a] The sorted CD 133+ cells were cultured in DMEM/199 media with 10% FBS.
[b] The sorted CD133+ cells were cultured as spheroids in DMEM/F12 media.

A recent study confirmed the significance of CD133 as a marker for both normal and tumor-initiating prostate cells [24]. Within several androgen receptor positive (AR+) human prostate cancer cell lines, CD133+ cells were found at low frequency and were able to self-renew, generate heterogeneous progenies and were capable of an unlimited proliferation capacity. The authors of this study also speculated that CD133 may function differently between normal and cancer prostate cells and that malignant CD133+ cells are originating from a malignantly transformed intermediate cell. Finally, it was confirmed that in addition to CD133+, the $CD44/\alpha_2\beta_1$ $integrin^{high}$/CD133+ population from the DU145 prostate cancer cell line [25] had high capacity of self-renewal and differentiation as well as strong proliferative and tumorigenic potential.

Another popular method to identify tumor-initiating cells is the isolation of the "side population" (SP). The SP cells are isolated based on the ability of cells to retain Hoechst dye, and in the LAPC-9 prostate cancer cell line the SP cells were shown to be more tumorigenic than the corresponding main population [26]. The LAPC-9 SP cells possessed other stem cell properties such as capacity of differentiation in vivo, as well as the ability to sustain subsequent transplantation. Additional information about stem cell surface makers (e.g. CD133, CD44, and $\alpha_2\beta_1$ integrin) was not provided by this study.

A different strategy adopted to identify tumor-initiating cells is based on their capacity to form holoclones — tightly packed clones with specific morphology that contain self-renewing cells and have been hypothesized to contain tumor-initiating cells [27]. The other two types of clones formed by epithelial cells (meroclones and paraclones) do not have the sustained proliferation capacity required for tumor initiation. Holoclones derived from the PC3 prostate cancer cell line were shown to contain stem-like cells that could initiate serially transplantable tumors [28]. In contrast, meroclones and paraclones did not proliferate and failed to initiate tumor development. Perhaps not surprising, the holoclones had high levels of CD44, $\alpha_2\beta_1$ integrin and β-catenin expression, whereas meroclones and paraclones show reduced expression of these stem cell markers. However, CD133 expression was not reported in this study.

In our experiments, we examined by immunofluorescence imaging the signature of DU145 prostate cancer cells grown at clonal density in attachment-independent conditions (spheroids). The attachment-independent conditions exert even more strain on the cells, and because spheroid formation was used extensively to enrich for stem cells, the clonogenic spheroid formation assay probably identifies the population of cells that have the highest tumorigenic potential. We specifically focused on common tumor-initiating cells markers such as CD44 and CD133. CD44 is present at high levels in the majority of DU145 cells, regardless of culture conditions (monolayer or spheroids). The CD133+ cells were also clearly identified in the spheroids,

but as the spheroids grew in size, the CD133+ population did not proliferate at the same rate as the CD44 cells within the spheroids (data not shown). While these spheroids were grown in serum-supplemented media (that usually promotes differentiation), the use of serum-free defined media also did not enrich the CD133+ population.

In summary, the study of prostate tumor-initiating cells is still an evolving field but the results to date suggest that a series of several surface and/or metabolic markers may be needed to identify prostate cancer-initiating cells.

## 4. Telomeres, telomerase and prostate cancer

Telomeres are nucleoprotein complexes that cap the ends of human chromosomes [29]. As the cells divide, the telomeres shorten by approximately 50–100 base pairs with each division [30]. In addition, single-strand breaks of telomere DNA caused by oxidative damage can lead to telomere attrition [31,32]. Telomeres present a specific end-replication problem, recognized as early as 1970's [33,34], and a specialized cellular enzyme, called telomerase is responsible for telomere extension [35]. Telomerase is active in proliferating cells of the skin, gastrointestinal system and blood [36–38]. While normal prostate cells lack telomerase activity [39], telomerase is detected in the majority of prostate cancer samples, being absent or present at low levels in benign prostate hyperplasia (BPH) [40–45]. More significant is the fact that majority of the prostate cancer samples have much shorter telomeres than the corresponding normal or BPH prostate samples [39,46].

Prostate cancer cells have robust telomerase activity and several commonly used prostate cancer cell lines show a significant TRAP signal (Fig. 1A). Similar to the results observed in primary tumor samples (data not shown), these cell lines have relatively short telomeres (Fig. 1B). The cell lines used were originally derived from different metastatic sites and cultured in serum-supplemented media, but primary prostate cancer cells cultured in our lab have shown the same characteristics (high telomerase activity and short telomeres) (data not shown). This is in contrast to normal prostate epithelial cells which are telomerase (TRAP) negative (Fig. 1A).

Androgen ablation in a rat model leads to activation of telomerase and further treatment with androgen reverses the effects and results in the down-regulation of telomerase in these animals [47]. Similar results were obtained in a primate model [48]. In sharp contrast, telomerase regulation by androgens is reversed in prostate cancers. Telomerase activity is up-regulated upon androgen stimulation and in clinical specimens telomerase activity is significantly reduced after complete androgen ablation [49,50]. Recent studies have shown that in prostate cancer cells the catalytic protein component of telomerase (hTERT) promoter is the target of down-regulation by AR in cooperation with p53 [51]. These experiments appear to reveal a mechanism for the protective role of androgens in normal prostate and suggest that prostate cancer cells might escape this mechanism by mutations in the AR. There is general agreement that normal adult prostate stem cells are AR+ but whether AR is expressed in tumor-initiating cells is still a controversial topic. While some authors support the idea that prostate tumor-initiating cells do not express AR [52,53], recent evidence supports the notion that prostate tumor-initiating cells are AR+ [24,54].

Chromosomal instability is an essential feature of prostate cancer, being detected as early as prostatic intraepithelial neoplasia (PIN), the earliest recognizable form of the disease. Using a high resolution, quantitative in situ method of investigation for telomere length [55], it was shown that the telomere length of high grade PINs were considerably shorter than adjacent normal cells [56]. The telomere shortening was restricted only to the luminal compartment, suggesting that the basal cells may not be the source of neoplastic transformation. This report supported previous studies showing that a subset of PIN cells activate telomerase, become immortal and eventually progress to fully invasive adenocarcinoma [41]. Combining this theory of neoplastic transformation with the evidence provided by the presence of AR in prostate tumor-initiating cells [24,54], we favor a model of prostate cancer in which telomere shortening and telomerase activity play a central role (Fig. 2). Under

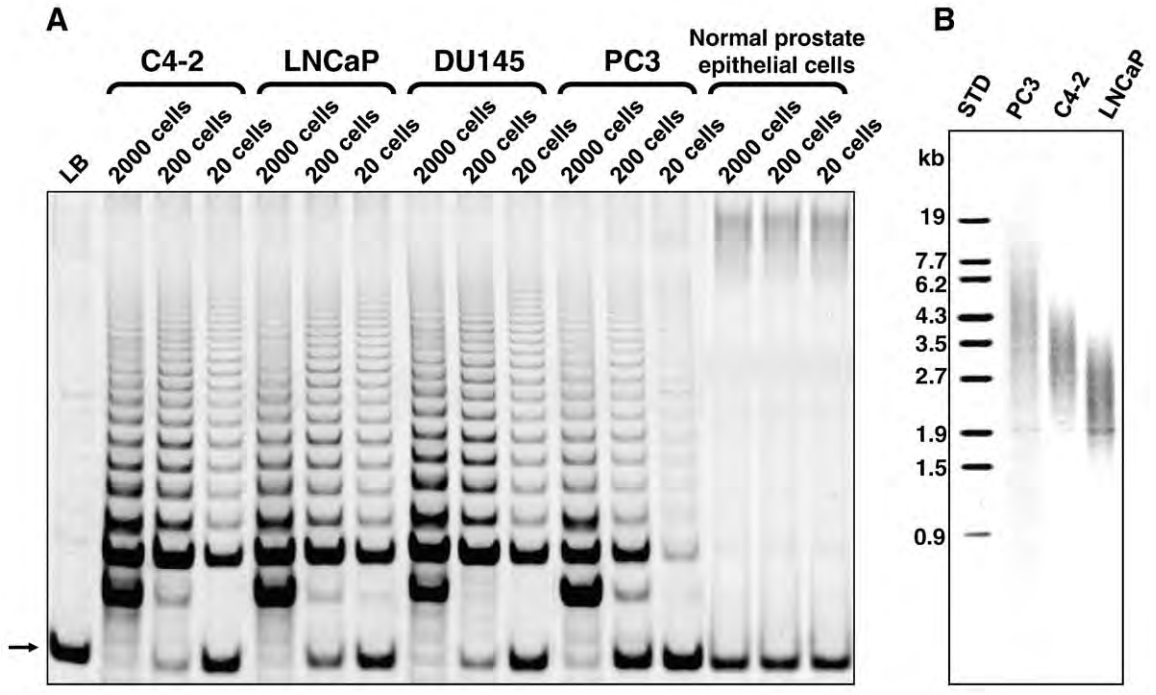


**Fig. 1.** Prostate cancer cell lines have telomerase activity and short telomeres. (A) Telomere Repeat Amplification Protocol (TRAP) on cell lysates from different prostate cell lines. Normal primary prostate epithelial cells are telomerase negative. The internal amplification standard used for quantifying telomerase activity levels is indicated by an arrow. (B) Terminal Restriction Fragment (TRF) assay on telomere DNA extracted from three different prostate cancer cell lines.

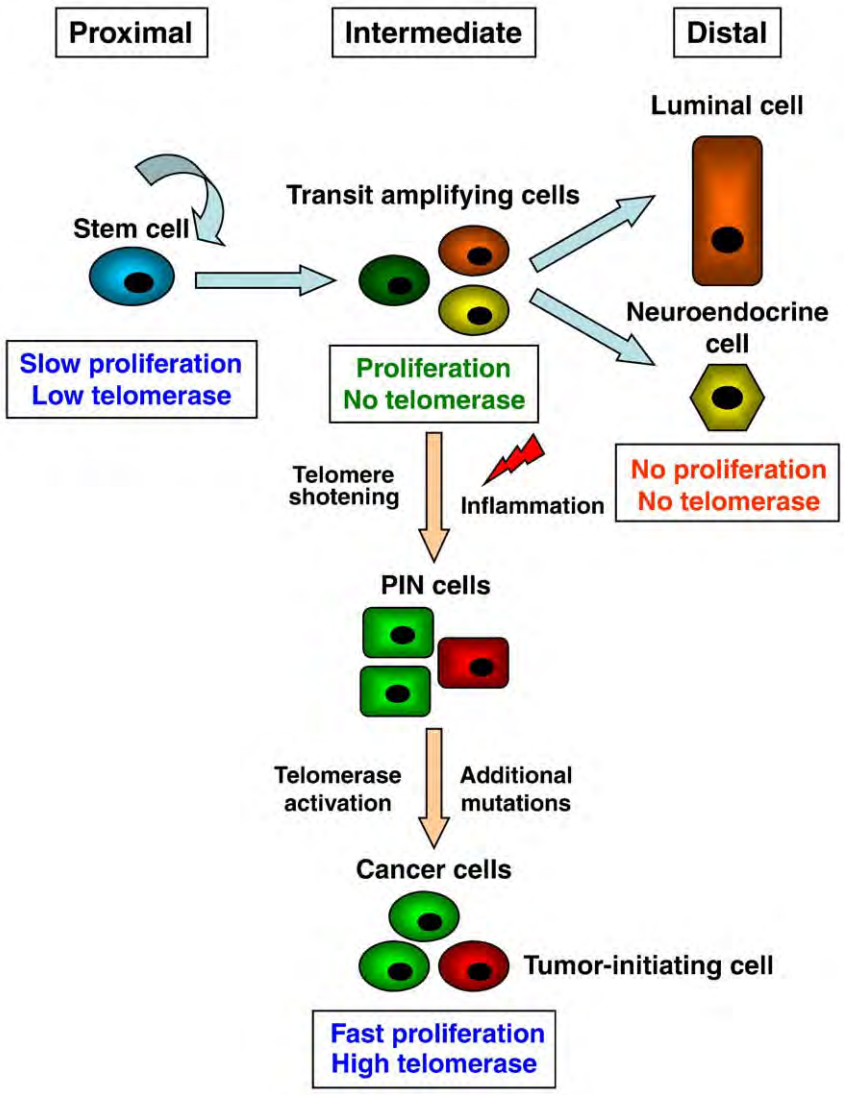


**Fig. 2.** Telomere dysfunction and telomerase activation play an important role in prostate malignant transformation.

the influence of chronic inflammation on a background of sustained telomere shortening, a subset of transit amplifying cells of intermediate phenotype will encounter severe chromosomal instability. To escape from the blockade induced by critically shortened telomeres, telomerase activation and subsequent cellular immortalization, combined with other mutations, leads to the development of prostate cancer. For example, cells in high grade PIN lesions that have escaped replicative senescence have unlimited proliferative capacity to accumulate additional mutations leading to prostate cancer.

## 5. Telomerase activity in prostate tumor-initiating cells

Telomerase in normal stem cells is highly regulated and generally expressed at low levels. The major function of the enzyme in the normal stem cell compartment is believed to be the partial maintenance of telomere homeostasis during self-renewal [57,58]. Importantly, all human adult stem cells (and normal somatic cells) that have been examined progressively shorten their telomeres with increased age. This telomere loss mechanism may have evolved as an important anticancer mechanism by placing limitations on cells that accumulated harmful mutations and preventing their clonal expansion. This suggests that fully maintaining telomere length in normal cells may increase the risk of developing cancer because cancer cells cannot enter replicative senescence and will ultimately achieve indefinite proliferation potential.

While there is no direct evidence about the levels of telomerase activity in prostate stem cells, the expectancy is that these cells will also have low levels of telomerase activity. The only experiments that addressed the telomerase activity in isolated tumor-initiating cells of solid tumors were performed in breast cancer, where the data presented shows that breast tumor-initiating cells have telomerase activity at similar levels with the main tumor mass cells, and importantly, that these cells have relatively short telomeres [59]. Experiments performed in our lab show that putative prostate tumor-initiating cells isolated from different cell lines have significant telomerase activity (Fig. 3). In the PC3 cell line, the surface markers CD44 and CD133 were used to isolate populations of cells that exhibit telomerase activity at the same level with the negative (low) fraction and main population. Two cell lines (LNCaP and PC3) that differ in terms of androgen response are shown for the CD133 marker, suggesting that telomerase is universally expressed in tumor-initiating cells isolated from cancer cell lines containing putative cancer-initiating markers. Experiments are under way to establish the presence of telomerase in tumor-initiating cells isolated from primary prostate tumor samples, as well as from distant metastatic sites in patients with advanced disease. We expect that prostate tumor-initiating cells isolated from these sources will have active telomerase,

these expectations being consistent with the properties of brain tumor-initiating cells (Marian et al., unpublished data).

## 6. Circulating prostate tumor-initiating cells

Metastasis is the major cause of death in patients with advanced prostate cancer, the most frequent site for metastatic lesions being the bone [60]. There are still many unanswered questions about the metastatic process, but for the purpose of this review we are interested in how the tumor-initiating cells and circulating tumor cells (CTC) fit in the general scheme of cancer progression. It was already hypothesized that a subset of tumor-initiating cells are responsible for distant metastasis [61,62]. Support for this hypothesis comes from the inherent properties of tumor-initiating cells. Only cells that have high plasticity and the capacity to form tumors will be responsible for the formation of metastatic lesions. These cells need to adapt to a new specific niche and the heterogeneous nature of tumor-initiating cells make them ideal candidates for this task. Detection of CTCs has improved with the advent of automated systems, some of which are approved for clinical use. One of these systems can be used as a survival predictor in patients with prostate metastatic cancer [63]. Very significant from the perspective of this review is the discovery that CTCs in the patients with advanced prostate cancer have significant levels of telomerase activity [64]. In the same study, telomerase activity was also detected in 23% of patient CTC specimens, all of which had undetectable serum PSA levels. This suggests the potential applications of this technique, not only for early diagnostic, but also for treatment monitoring. If circulating tumor cells contain small populations of tumor-initiating cells, it becomes important to determine if these cells are telomerase positive. If telomerase activity is maintained in tumor-initiating cells after dissemination, these cells will most likely have significant levels of telomerase activity. If these cells are telomerase negative, the up-regulation of telomerase activity will most likely occur after the initial period of quiescence commonly associated with disseminated cancer cells. Nevertheless, without telomere maintenance by telomerase, metastatic lesions cannot proliferate to a significant level that makes them dangerous for the patient. The presence of telomerase in prostate CTCs can also serve as a direct monitoring tool for telomerase inhibition therapy. The access to blood samples is more feasible and less invasive than the alternative bone marrow biopsies.

## 7. Telomerase as a therapeutic target for prostate cancer

Telomerase is also an attractive target for cancer therapy. The enzyme is present in majority of cancer cells analyzed but absent in almost all normal somatic cells, making telomerase inhibitors highly specific and telomerase a universal oncology target. Moreover, because normal cells have longer telomeres compared to cancer cells, the toxicity of these inhibitors in normal tissues is minimal. Several strategies have been employed for targeting telomerase, and several reviews have been written on the subject in recent years [65,66]. Despite a multitude of pre-clinical studies, only two of these strategies have led to drugs that are currently in clinical trials. The first strategy targets the functional RNA (*hTR* or *hTERC*) component of the telomerase enzyme with N3′–P5′ thio-phosphoramidate oligonucleotides [67]. The 13-mer compound used in these studies, GRN163L, is an antagonist that has high affinity to the *hTR* sequence and acts as an enzymes inhibitor (not as an antisense approach targeting mRNA). Once GRN163L is bound to the *hTR* component, it blocks access of *hTERT* (the catalytic protein component of telomerase) and prevents the assembly of an active telomerase enzyme. This leads to telomerase inhibition and progressive telomere shortening, eventually leading to telomere uncapping and cell death. GRN163L is currently in Phase I and I/II clinical trials in several hematological and solid tumor malignancies. While not tested specifically for prostate cancer in clinical trials, this telomerase antagonist along with its un-lipidated precursor (GRN163) was shown to be effective in prostate xenograft models, and thus may become an effective therapy for prostate cancer [68–70].

A second strategy employs active telomerase immunotherapy directed towards the hTERT catalytic component. The presence of telomerase-specific cytotoxic T lymphocytes has been discovered in some patients, suggesting that the immune system can elicit a response to telomerase-presenting cells even in the absence of vaccination [71]. In a clinical trial initiated in patients with prostate

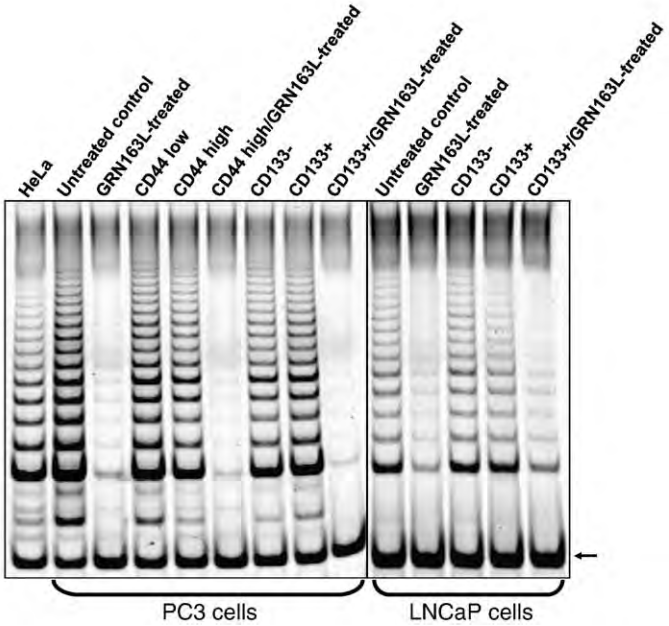


**Fig. 3.** Putative prostate tumor-initiating cells have telomerase activity which can be inhibited by telomerase inhibitors (GRN163L). Different populations of tumor-initiating cells were isolated by flow cytometry using surface markers (CD44 and CD133) and equal cell lysate amounts were used for the TRAP assay. The cells were pre-treated for 72 h with the telomerase inhibitor drug (2 μM) before sorting. HeLa cells were used as positive controls. The internal amplification standard is indicated by an arrow.

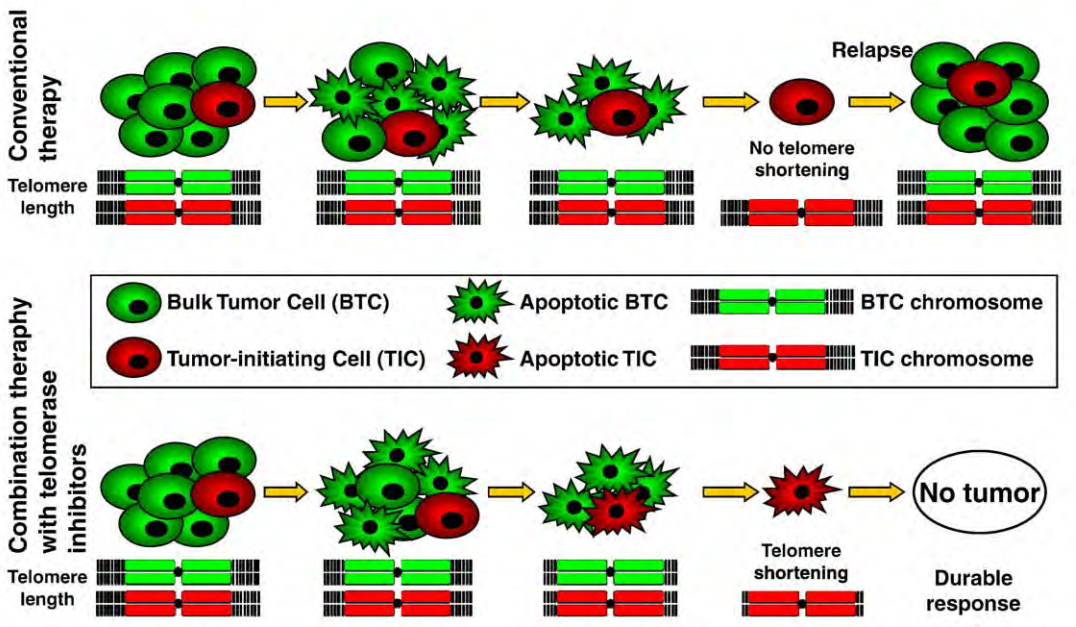


**Fig. 4.** Telomerase inhibitors used in combination with standard therapies can target tumor-initiating cells. Tumor-initiating cells escape conventional therapies and are responsible for relapse but telomerase inhibition can target all the tumor cell fractions and lead to a durable response.

metastatic cancer, the subjects were inoculated with dendritic cells transfected with an mRNA encoding a chimeric lysosome-associated membrane protein-1 (LAMP) hTERT protein which allows a concomitant CD8+ and CD4+ T cell response. For the patients involved in this study, the vaccine had a significant impact on PSA levels and also led to a transient elimination of the PSA-expressing circulating tumor cells [72]. Another clinical study with prostate cancer patients revealed a significant induction of hTERT-specific T lymphocytes in response to inoculations with dendritic cells pulsed with a HLA-A2-restricted hTERT I540 peptide and keyhole limpet hemocyanin (KLH) [73]. Both these studies, along with clinical trials initiated for other malignancies [74] have shown minimal side-effects and no adverse effects on normal bone marrow stem cells. These cancer vaccination studies appear very promising, and some of the telomerase vaccines are moving forward to Phase II and III clinical trials.

## 8. Telomerase inhibition in prostate tumor-initiating cells

Previous reports have shown that GRN163L inhibits telomerase activity and eliminates the clonogenic potential of tumor-initiating cells from several multiple myeloma (MM) cell lines. The same results were observed when using primary clinical samples, where tumor-initiating cells isolated from the bone marrow of patients with MM were exposed to GRN163L [75]. In our lab we established the presence of active telomerase in putative prostate tumor-initiating cells isolated from prostate cell lines and we proceeded to show that the telomerase inhibitor GRN163L is able to inhibit the enzymatic activity in these cells with the same efficiency as in the main population (Fig. 3). Telomerase inhibition induced progressive telomere shortening in tumor-initiating cells at the same rate found in the cell line from which they were derived (data not shown). After the telomeres become critically short, the cells will enter apoptosis and die (data not shown). These experiments suggest that GRN163L may be a valuable therapy for the treatment of prostate cancer, its unique mode of action being able to target the elusive putative tumor-initiating cells that are usually resistant to conventional therapies.

Assuming the same telomere shortening dynamics are maintained in vivo, we propose a therapy regimen that combines conventional approaches (surgery and chemotherapy/radiotherapy) with telomerase inhibitors (Fig. 4). While the standard therapy will have a debulking effect relatively fast, sustained telomerase inhibition will lead to critical telomere attrition and ultimately cell death in the small populations of cells that survive the first therapeutic intervention, including the tumor-initiating cells. The effect of this drug on normal stem cells in the organism should be minimal due to their slower proliferation rates, lower telomerase activity, and longer telomeres. This should provide an ample therapeutic window in which the shorter telomere-bearing tumor cells will be eliminated. After the telomerase inhibitor drug is removed, telomerase activity will return to normal levels in the proliferating cells.

Direct telomerase inhibitors are not the only agents that can be used with this therapy strategy, and vaccines targeting telomerase positive cells should be equally efficient, unless tumor-initiating cells have special mechanisms to escape detection by the immune system. Preliminary experiments from clinical trials show that the vaccine might target and eliminate the cancer cells found in circulation [73], but based on the available data there is no direct evidence that the vaccines are targeting specifically tumor-initiating cells. This brings up a very important issue related to the availability of biomarkers to monitor telomerase inhibition therapy in prostate cancer. Because telomerase inhibitors will act promiscuously on all the cells in the organism, several cell types are available to assess therapy efficiency. The less invasive approach makes use of peripheral mononuclear blood cells (PBMCs). Activated leukocytes have low but detectable telomerase activity, therefore telomerase inhibition in this compartment can be used in various pharmacodynamic studies [76]. A more direct approach is to measure telomerase activity in CTCs or in disseminated cancer cells isolated from bone marrow aspirates.

## 9. Concluding remarks

The origin and identification of tumor-initiating cells is an exciting area of research, full of possibilities but also controversies that generate vivid arguments. The majority of these arguments are generated by the theoretical concepts associated with the “cancer stem cell” hypothesis and by some technical issues that make the isolation and characterization of these cells problematic [77,78]. Some of these concerns are valid and more investigations are required to address these issues. However, the most valuable application of this scientific knowledge should be the discovery of new therapeutic

strategies for the treatment of cancer. Telomerase inhibition might be one of these novel targeted therapies, and due to the fundamental role of telomerase in most malignancies, we propose that telomerase therapeutics may also target tumor-initiating cells. If the source of tumor initiation is eradicated by targeting the ability of cell to maintain the end of the chromosomes, the quest for a cure might also come to an end.

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# The effects of telomerase inhibition on prostate tumor-initiating cells

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**Prostate cancer is the most common malignancy in men, and patients with metastatic disease have poor outcome even with the most advanced therapeutic approaches. Most cancer therapies target the bulk tumor cells, but may leave intact a small population of tumor-initiating cells (TICs), which are believed to be responsible for the subsequent relapse and metastasis. Using specific surface markers (CD44, integrin $\alpha_2\beta_1$ and CD133), Hoechst 33342 dye exclusion, and holoclone formation, we isolated TICs from a panel of prostate cancer cell lines (DU145, C4-2 and LNCaP). We have found that prostate TICs have significant telomerase activity which is inhibited by imetelstat sodium (GRN163L), a new telomerase antagonist that is currently in Phase I/II clinical trials for several hematological and solid tumor malignancies. Prostate TICs telomeres were of similar average length to the telomeres of the main population of cells and significant telomere shortening was detected in prostate TICs as a result of imetelstat treatment. These findings suggest that telomerase inhibition therapy may be able to efficiently target the prostate TICs in addition to the bulk tumor cells, providing new opportunities for combination therapies.**



Early detection combined with androgen depletion therapy significantly reduces morbidity in patients with localized prostate cancer, but for the patients with metastatic disease the therapeutic options are limited.[1] The development of castrate- and drug-resistant tumors poses further challenges in the treatment of prostate cancer.[2] Therefore, understanding the etiology of prostate cancer may lead to the development of new chemotherapeutic agents, which can circumvent the limitations of current therapies.

The initial tumor formation and subsequent tumor relapse are believed to be caused by small populations of cells, known as tumor-initiating cells (TICs) or cancer stem cells.[3,4] The existence of TICs was suggested by the observation that cancers are composed of heterogeneous cell populations, with different capacities of tumor initiation.[5,6] According to this hypothesis, targeting the TICs may be the only viable method to eliminate the tumor and achieve a significant therapeutic response. Several experimental strategies have been used to identify prostate TICs. One of the most popular strategy uses specific surface markers such as CD44,[7–13] integrin $\alpha_2\beta_1$,[14,15] CD133[16] or a combination of the above.[17–19] A different approach is to isolate side population (SP) cells based on the exclusion of Hoechst 33342 dye.[20,21] Finally, an innovative strategy is based on the hypothesis that only the holoclones (tightly packed round colonies of cells with distinct morphology) are able to re-initiate tumor growth.[22,23]

Telomeres are specialized nucleoprotein complexes that protect the ends of linear chromosomes[24] and in the vast majority of human tumors telomere lengths are maintained by telomerase.[25] Previous studies have shown that almost all prostate carcinomas have detectable telomerase activity,[26–30] and there is a direct correlation between the total amount of telomerase and the Gleason score.[31,32] By contrast, in normal prostate tissues, telomerase activity is absent.[33] The increased level of telomerase activity almost universally present in carcinomas and the lack of telomerase in most normal tissues make it an attractive target for anticancer therapy.[34–40] One of the most efficient telomerase inhibitors is a N3′-P5′ *thio*-phosphoamidate oligonucleotide antagonist (GRN163, Geron Corporation, Menlo Park, CA), which causes telomerase inhibition and progressive telomere shortening in numerous cancer cell types.[40–43] The second generation of GRN163, designated imetelstat sodium (GRN163L), shows increased intracellular uptake, increased telomerase inhibition and telomere shortening in several cancer cell lines.[44–47] Imetelstat has now entered early stage clinical trials as single agent for chronic lymphocytic leukemia and multiple myeloma and in combination with standard chemotherapeutics for non-small cell lung cancer and breast cancer.[48]

We previously hypothesized that telomerase inhibition can efficiently target the TICs,[49] but there are few rigorous scientific investigations that study the telomere biology of TICs. It is generally believed, but not well documented, that TICs are

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telomerase-positive, but little is known about the telomere length of these cells.[50] In this study, we set out to investigate if prostate TICs have telomerase activity and if these cells could be efficiently targeted by telomerase inhibitor drugs such as imetelstat. This report demonstrates that prostate TICs have high levels of telomerase activity and that treatment with imetelstat leads to telomerase inhibition and subsequent telomere shortening in the prostate TICs. These results have important therapeutic implications for telomerase inhibitor drugs in prostate cancer therapy.

## Material and Methods

### Cell lines

The prostate cancer cell line DU145 was maintained in a 4:1 mixture of Dulbecco's modified Eagle's medium and medium 199 supplemented with 10% cosmic calf serum (HyClone, Logan, UT). The PC3, C4-2 and LNCaP prostate cancer cell lines were grown in T-medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal calf serum (HyClone, Logan, UT). All the cell lines were kept in a humidified incubator with 5% $CO_2$, at 37°C.

### Imetelstat treatment

Imetelstat (5′-Palm-TAGGGTTAGACAA-NH2-3′) is an oligonucleotide containing a sequence complementary to the hTR template region of telomerase. For short-term telomerase inhibition, the cells were treated with 1 μM drug 72 h prior to TIC isolation. For long-term treatment, leading to telomere shortening, the cells were passaged weekly and treated with 2 μM drug every 3 days.

### Isolation of TICs using surface markers

Cells were grown on 15-cm tissue culture dishes (BD Falcon, Bedford, MA) until they became subconfluent, then gently detached using 0.05% Trypsin EDTA (Invitrogen, Carlsbad, CA). After detachment, the total number of cells was determined using a Z1 Coulter Counter (Beckman Coulter, Fullerton, CA) and the cells were resuspended in cold 1× PBS at a density of $1 \times 10^7$ cells/100 μl. The following antibodies and dilutions were used: 1:10 integrin alpha 2 (AK7) mouse monoclonal FITC-conjugated antibody (Abcam, Cambridge, MA), 1:10 CD44 (G44-26) mouse monoclonal PE-conjugated antibody (BD Biosciences, San Jose, CA) and 1:10 CD133 (AC133) mouse monoclonal PE-conjugated antibody (Miltenyi Biotec, Auburn, CA). The cells were incubated with the antibodies on ice for 20 min, then washed twice with cold 1× PBS. After washes, the cells were strained through a nylon mesh (70-μm cell strainer, BD Falcon, Bedford, MA) and maintained on ice until FACS analysis. Cell sorting was performed on a Becton-Dickinson FACSAria (BD Biosciences, San Jose, CA). IgG samples were used as negative controls and the positive cells gated out of the living cell population. Tumor-initiating fractions were sorted for both controls and imetelstat treatment groups.

### Isolation of SP (Side Population)

The SP protocol was based on Goodell *et al.*[51] The cells ($1 \times 10^6$/ml) were incubated in warm T-medium with 5% fetal bovine serum containing 5 μg/ml Hoechst 33342 for 1 h at 37°C with occasional mixing. A control sample was incubated with 50 μM verapamil to confirm the nature of the SP. After incubation, the cells were resuspended in cold 1× PBS and propidium iodide was added to a final concentration of 2 μg/ml before FACS analysis. The samples were analyzed on a MoFlo flow cytometer (Beckman Coulter, Fullerton, CA) with UV excitation at 360 nm. The fluorescence was measured with a 670-nm filter and a 405-nm filter.

### Isolation of holoclones and spheroid formation assays

Cells were plated low density (500 cells/10-cm dishes) and after 10 days the colonies were counted and holoclones were isolated based on their morphology using small diameter cloning rings. Holoclones are tightly packed colonies of small cells with round morphology; meroclones possess an intermediate phenotype and paraclones have irregular shape and are composed of large, loosely packed cells. The clones were briefly expanded, then harvested for subsequent analysis. For the assessment of clonogenicity in long-term imetelstat-treated cells, we used both serial dilutions in 96-well plates and 10-cm dishes. For the clonogenic spheroid formation assays, the cells were plated on ultra-low attachment dishes (Corning Life Sciences, Lowell, MA) and the spheroids were counted after 10 days of culture.

### Telomerase activity

The telomerase activity was measured using a Telomeric Repeat Amplification Protocol (TRAP) with the TRAPeze kit (Chemicon, Temecula, CA) according to the manufacturer's instructions. The cells were pelleted, lysed in CHAPS buffer (on ice) and after preparing the PCR reactions with cell lysates equivalent to equal number of cells, the telomerase extension products were amplified using a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, MA). The samples were resolved on a 10% polyacrylamide gel and visualized using a Typhoon Trio Variable Mode Imager (Amersham Biosciences, Piscataway, NJ). The telomerase products (6-bp ladder) and the 36-bp internal control (ITAS) bands were quantified using the AlphaImager 2000 software (Alpha Innotech, San Leandro, CA). The relative telomerase activity (RTA) was calculated as the intensity ratio of the TRAP ladder to that of the ITAS band, and the relative intensity of each sample was normalized to that of the positive control.

### Telomere length

Total DNA was extracted from the cancer cells using the DNeasy Blood and Tissue Kit (Qiagen Sciences, MD). Telomere restriction fragment (TRF) analysis was performed as described previously.[46] Briefly, 1 μg of total DNA was digested with a mixture of 6 enzymes and separated on an

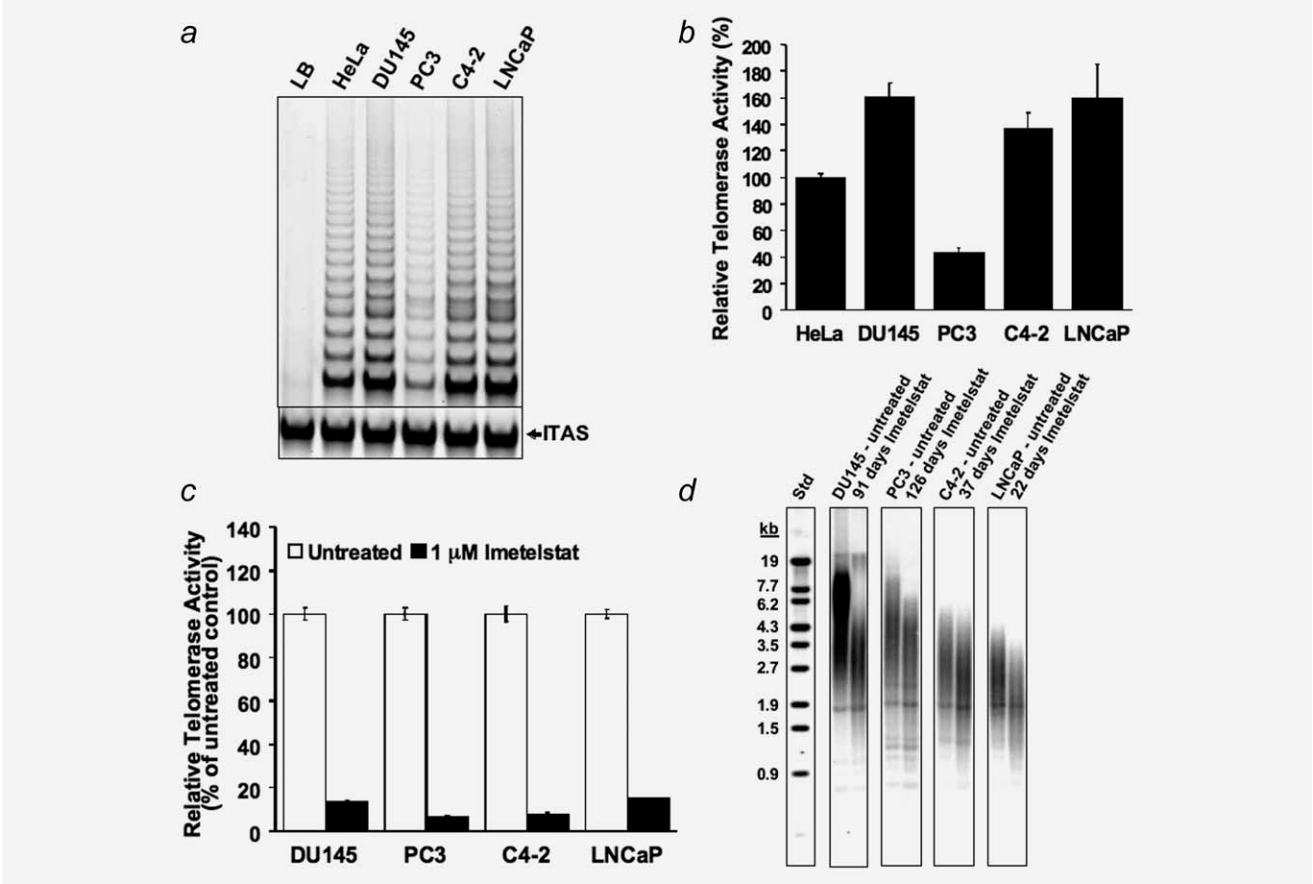


**Figure 1.** Prostate cell lines have high levels of telomerase activity which can be inhibited by imetelstat. (*a*) Telomeric repeat amplification protocol (TRAP) assay of 4 prostate cancer cell lines compared with the HeLa cells. (b) Quantification of the TRAP signal presented as a ratio between the intensity of the telomerase ladder signal *versus* the intensity of internal amplification standard (ITAS) band. (*c*) Imetelstat (1 μM) inhibits telomerase activity efficiently in all the cell lines analyzed. Relative telomerase activity (RTA) was normalized to the untreated control cells. (*d*) TRF (telomere analysis) shows that sustained telomerase inhibition with 2 μM imetelstat leads to telomere shortening. Lysate equivalent to the same number of cells was used for TRAP with all the cell lines.

agarose gel. The gel was denatured, dried and neutralized in 1.5 M NaCl and 0.5 M Tris–HCl at pH 8.0. The gel was then hybridized with a $32^{P}$-labeled telomeric probe overnight at 42°C. After several washes, the gel was exposed to a Phosphor screen overnight, which was analyzed using a Typhoon Trio Variable Mode Imager (Amersham Biosciences, Piscataway, NJ).

## Results

### Inhibition of telomerase activity in prostate cancer cell lines by imetelstat leads to telomere shortening

First, we set out to evaluate the effects of imetelstat on the whole population of prostate cancer cells. Figure 1*a* shows the expected 6-bp TRAP ladder for 4 different prostate cancer cell lines (DU145, PC3, C4-2 and LNCaP), quantified in Figure 1*b*. All the cell lines used in this study have significant levels of telomerase activity, 3 of them (DU145, C4-2 and LNCaP) have more RTA (relative telomerase activity) when compared to HeLa cells. Treatment with imetelstat leads to efficient telomerase inhibition (Fig. 1*c*) in a dose-dependent fashion (gel data not shown). Prolonged telomerase inhibition due to imetelstat treatment leads to telomere shortening in all the prostate cancer cell lines analyzed (Fig. 1*d*). The telomere lengths of the cells used in our experiments vary in average size, from short (LNCaP) to relatively long (DU145), and there is no correlation between telomere length and telomerase activity. If telomerase inhibition (2 μM every 3 days) was maintained until the telomeres became critically short, the cells ceased to proliferate and ultimately died (data not shown). More importantly, there is a correlation between the interval of time required for the onset of apoptosis as a result of telomere shortening (due to imetelstat treatment) and the initial telomere length.

### Prostate TICs isolated using established surface markers are telomerase-positive and sensitive to telomerase inhibition by imetelstat

It was previously shown that DU145 CD44+/integrin $\alpha_2\beta_1^{hi}$ cancer cells possess traits of tumor stem/progenitor cells and

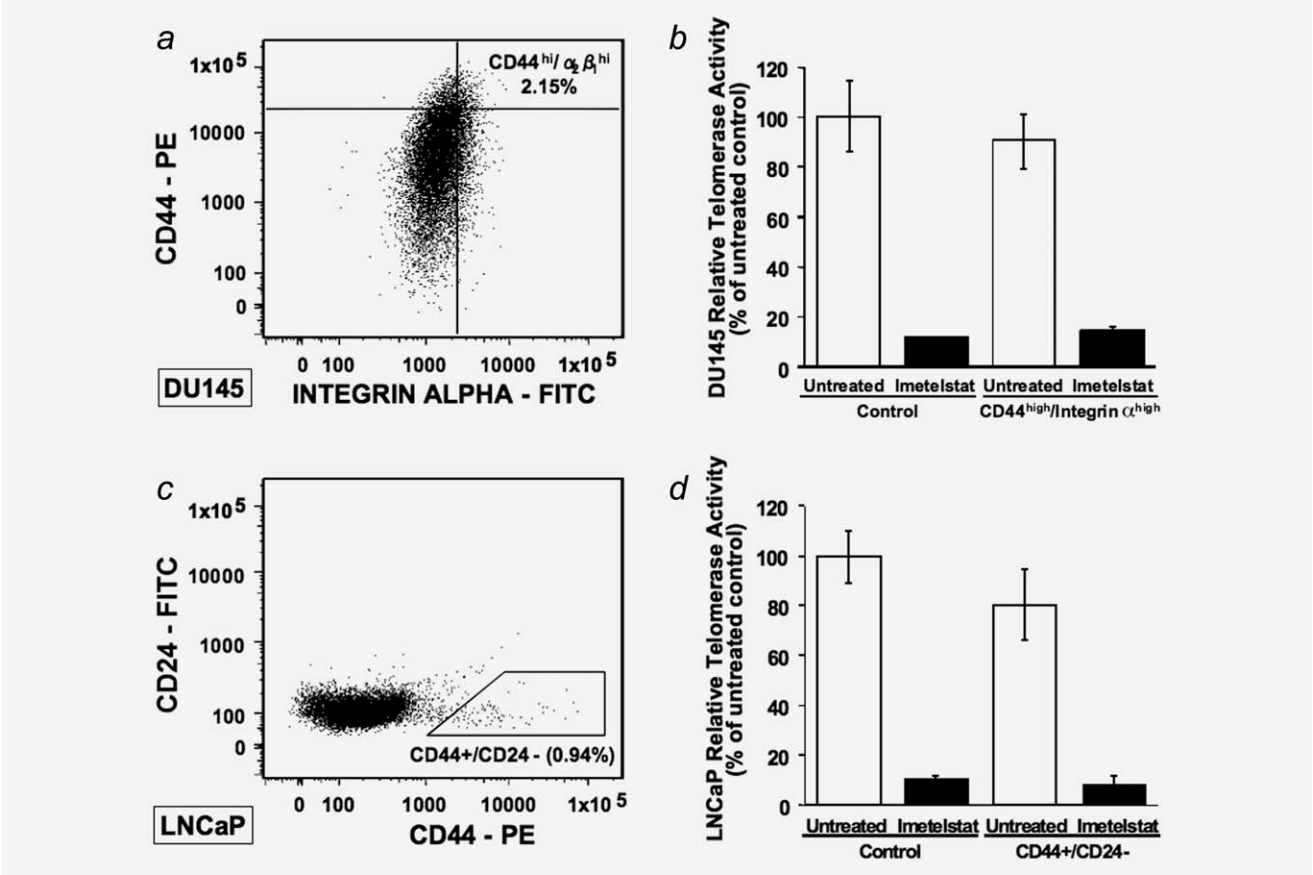


**Figure 2.** Imetelstat acts efficiently on prostate TICs isolated using the CD44 surface marker. (*a*) DU145 cells with the CD44$^{hi}$/integrin $\alpha_2\beta_1{}^{hi}$ phenotype were isolated using fluorescence-activated cell sorting (FACS). (*b*) RTA in the untreated and imetelstat-treated cells for total and CD44$^{hi}$/integrin $\alpha_2\beta_1{}^{hi}$ cells. (*c*) LNCaP cells possess a small population of CD44/CD24− cells as illustrated by FACS. (*d*) RTA in the untreated and imetelstat-treated cells for total and CD44+/CD24− cells. The cells were treated with 1 μM imetelstat before sorting and analysis.

are more proliferative, clonogenic, tumorigenic and metastatic than the CD44+/integrin $\alpha_2\beta_1{}^{low}$ cells.[15] We sorted the top 10% cells stained with each antibody, which translated to ~2% of the total population (Fig. 2*a*). Equal numbers of CD44$^{hi}$/integrin $\alpha_2\beta_1{}^{hi}$ cells were collected for the untreated and imetelstat-treated samples and subsequently used for the TRAP assay. The DU145 CD44$^{hi}$/integrin $\alpha_2\beta_1{}^{hi}$ cells have telomerase levels similar to that of total population, and imetelstat inhibition of telomerase activity is equally efficient in this putative stem/progenitor cell fraction (Fig. 2*b*).

In the LNCaP cell line, the isolated CD44+/CD24− population is highly tumorigenic and expresses specific genes known to be important in stem cell maintenance.[12] Consistent with this previous study, the percentage of LNCaP cells that stained positive for CD44 was relatively small, less than 1% (Fig. 2*c*). The LNCaP CD44+/CD24− cells had high levels of telomerase activity, which was similar to the main population of LNCaP cells. Again, imetelstat was able to robustly inhibit telomerase activity in both fractions (Fig. 2*d*).

CD133+ cells isolated from the DU145 line have the capacity of self-renewal and differentiation, as well as high proliferative and tumorigenic potential.[19] An antibody against CD133 (prominin-1) was used to sort a small population of CD133+ cells from the DU145 line (Fig. 3*a*). DU145 CD133+ cells have high levels of telomerase activity, similar to the levels found in the total population of cells, and imetelstat is effective at inhibiting telomerase in these putative stem-like cells (Fig. 3*b*).

These results show that populations of prostate TICs isolated using most of the surface markers cited in the literature are telomerase-positive and are sensitive to the telomerase inhibitor imetelstat.

### The SP (side population) cells have high levels of telomerase activity which is inhibited by imetelstat

The SP, believed to harbor the TICs, can be isolated by sorting cells which exclude the Hoechst 33342 dye.[21,52] Out of the 4 cell lines analyzed, we detected a small SP only in the C4-2 prostate cell line (an LNCaP derivative), which accounted for ~0.1% of the total population (Fig. 3*c*). TRAP assays show that the SP cells isolated from the C4-2 cells have high levels of telomerase activity, slightly higher than

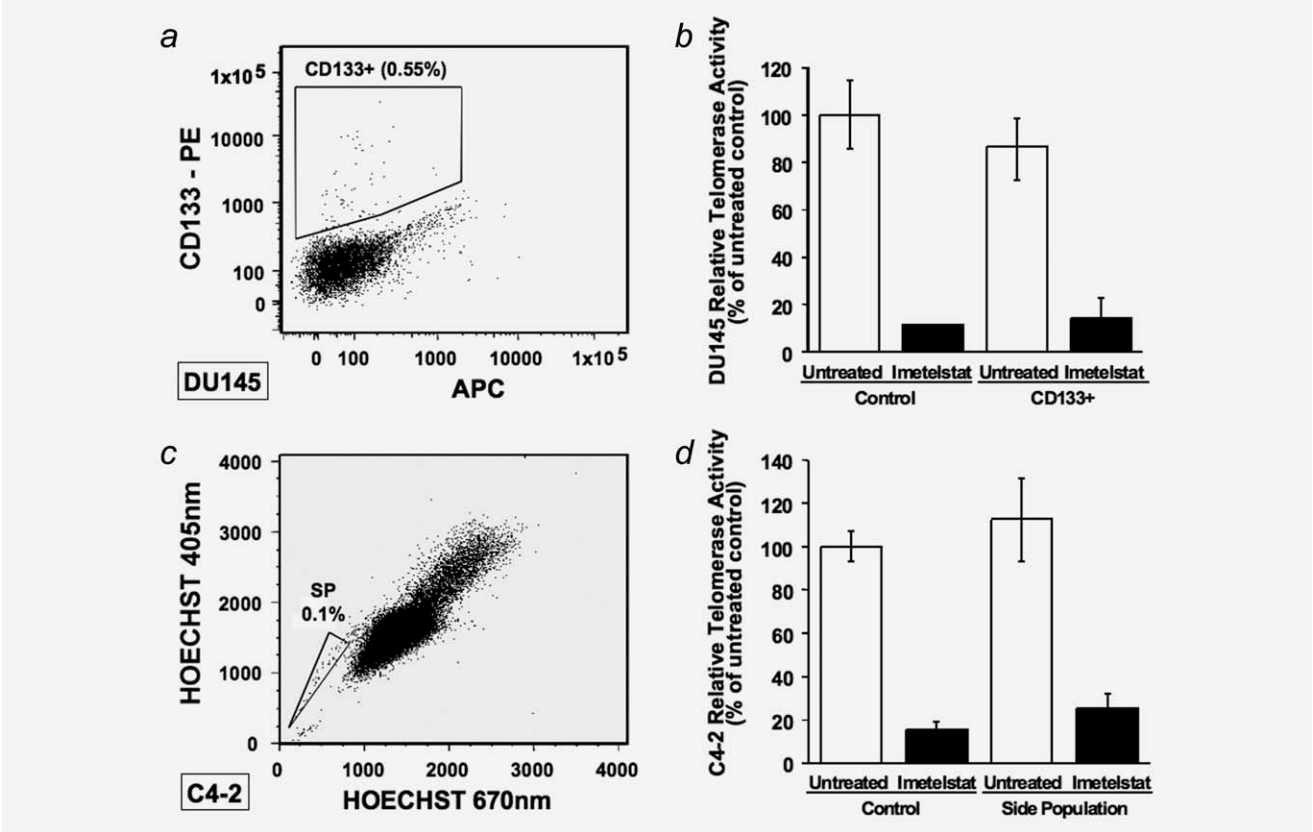


**Figure 3.** CD133+ and SP cells are telomerase-positive and sensitive to imetelstat. (*a*) DU145 cells contain a small population of CD133+ TICs which were isolated using FACS. (*b*) RTA in DU145 CD133+ cells compared to the total population of cells. Imetelstat inhibits telomerase activity in the CD133+ cell population. (*c*) The C4-2 SP was isolated based on Hoechst 33342 dye exclusion. (*d*) C4-2 SP cells have similar telomerase activity to the main population of cells, and imetelstat-mediated telomerase inhibition is equally efficient in both fractions. The cells were treated with 1 μM imetelstat before sorting and analysis.

the main population of cells, and the enzyme's activity was inhibited efficiently by imetelstat (Fig. 3*d*).

### The average telomere length of sorted prostate TICs is similar to the telomere size found in the main population of cells

Once we established that the prostate TICs have significant levels of telomerase activity and that telomerase inhibitors can target efficiently these cells, we investigated the average telomere lengths of prostate TIC fraction. One possibility was that TICs may have longer telomeres compared to the bulk population of cells. As seen in Figure 4, telomeres of prostate TICs are generally of similar average size with the main population of cells from which they were isolated. While the average telomere lengths in TICs *versus* main population do not vary significantly, the distribution of the telomere length might be different. This is important because the various methods of TICs isolation currently used may not identify cells with identical phenotype. As seen in Figure 1*d*, the DU145 cells had 2 relative distinct populations of cells, one with long telomeres (~6.5 kb), which produce the most intense smear on the TRF gel, and the other with shorter telomeres (~3.4 kb), which produce a smear of lower intensity. In Figure 4*b*, for the untreated control, only the larger size subpopulation of telomeres was visible due to the low amount of DNA loaded, but for the $CD44^{hi}$/integrin $\alpha_2\beta_1{}^{hi}$ cells, both populations of telomeres were visible at similar intensities. By contrast, for the DU145 CD133+ TICs, this phenomenon was not apparent; only the large fraction of telomeres was visible on the TRF gel.

The similar average telomere size of TICs and main population of cells would predict that telomere attrition should occur at equal rates in these cells. To verify this hypothesis, we isolated TICs from cell cultures treated with imetelstat for longer periods of time. As illustrated in Figure 4, the telomerase inhibitor was able to induce telomere shortening in these cells, and it appears that the rate of telomere shortening in these cells is similar to that found in the main population of cells.

### DU145 prostate cancer holoclones have significant levels of telomerase activity and relatively short telomeres

It has been reported that out of the 3 types of clone morphology (holoclone, meroclone and paraclone) formed by some prostate cancer cell lines, only the cells which form

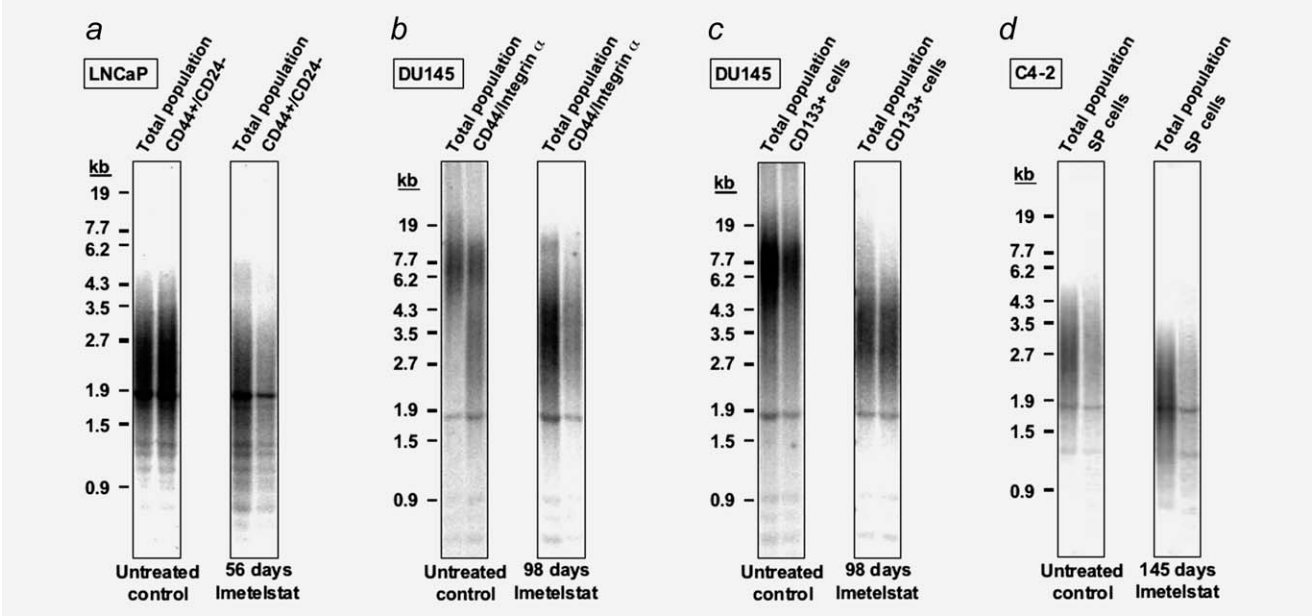


**Figure 4.** Telomere lengths in prostate TICs compared with the telomeres of untreated and imetelstat-treated cells. (*a*) LNCaP cells were treated with 2 μM imetelstat for 56 days, then the CD44+/CD24− cell fraction was isolated and TRF assay performed on the extracted genomic DNA. (*b*) DU145 cells were treated with imetelstat for 98 days, then the $CD44^{hi}$/integrin $\alpha_2\beta_1{}^{hi}$ population was isolated by FACS and TRF performed on total genomic DNA extracted from the cells. (*c*) TRF on total genomic DNA extracted from DU145 CD133+ cells treated with imetelstat for 98 days was compared with the telomere signal obtained from the untreated total population of cells. (*d*) The C4-2 SP is sensitive to telomere erosion effects of imetelstat similarly to the main population of cells.

holoclones (tightly packed small cells) are capable of extensive proliferation and tumor initiation in immunocompromised mice.[22,23] By contrast, meroclones (intermediate phenotype) and especially paraclones (loosely packed large cells) have reduced proliferation and tumorigenic potential. Holoclones are enriched in TICs, and isolation of these populations of cells does not require the use of surface markers in conjunction with FACS or magnetic beads. We used very stringent criteria to isolate several DU145 holoclones (Fig. 5*a*) and expanded them briefly in culture, just long enough to harvest enough material for the subsequent assays. As illustrated in Figure 5*b*, telomerase activity in these holoclones was on average only slightly lower than that in the DU145 total population. Moreover, the telomere lengths of 7 DU145 holoclones were shorter than the main population of cells (Fig. 5*c*), suggesting that these cells will be susceptible to telomerase inhibition treatment.

### Long-term treatment with imetelstat may reduce the number of TICs and lead to a decreased capacity of self-renewal in prostate cancer cell lines

When we compared the FACS profile of cells treated for different periods of time with imetelstat, we observed that the proportion of DU145 $CD44^{hi}$/integrin $\alpha_2\beta_1{}^{hi}$ cells present in the total population decreased proportional with the length of imetelstat treatment (Fig. 6*a*). Using identical gating criteria on the FACS plots, it was evident that the number of $CD44^{hi}$/integrin $\alpha_2\beta_1{}^{hi}$ cells decreased dramatically after prolonged imetelstat treatment. This phenomenon was observed only after some telomere shortening occurred, because short-term imetelstat-treated cells have the same percentage of $CD44^{hi}$/integrin $\alpha_2\beta_1{}^{hi}$ cells as in the untreated population (data not shown). To further investigate if imetelstat treatment gradually eliminates TICs from the population, we proceeded to examine the capacity of imetelstat-treated cells to generate holoclones. As illustrated in Figure 6*b*, after ~100 days of treatment, we were unable to detect any holoclone formation when the cells were plated at low density. As expected, the numbers of paraclones increased, probably due to the cells with the shortest telomeres in the population entering replicative senescence, but using our scoring criteria no significant differences in the number of meroclones were observed.

Because the spheroid formation assay is an indicator of self-renewal capacity, we examined whether long-term treatment with imetelstat has an effect on the capacity of self-renewal at the population level. The clonogenic spheroid formation assay showed that prolonged treatment with imetelstat leads to decreased spheroid formation ability in all of the cell lines analyzed (Fig. 6*c*), supporting the hypothesis that the TICs are gradually eliminated from the population.

## Discussion

Telomerase expression (limitless proliferation) is one of the hallmarks of cancer, and several experimental therapeutic approaches have focused on exploiting this almost universal

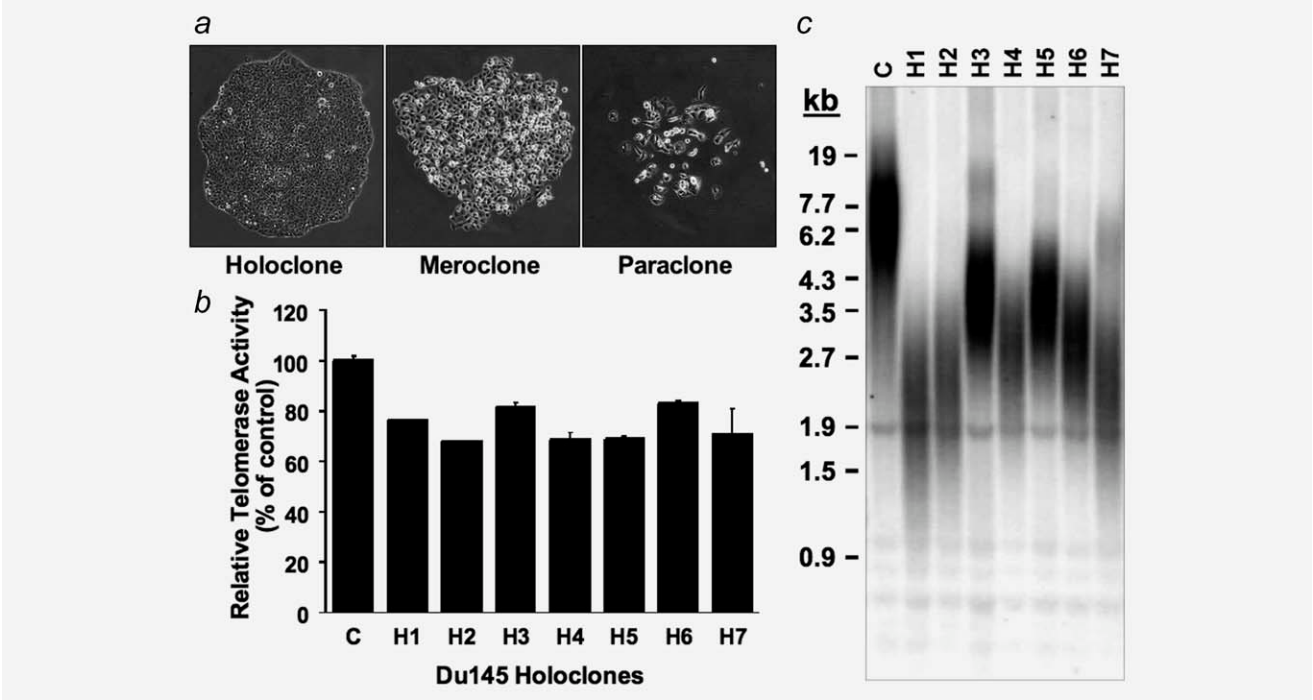


**Figure 5.** DU145 holoclones have significant levels of telomerase activity, and the average telomere length of these cells is shorter than the main population of cells. (*a*) Characteristic morphology of DU145 clones; holoclones can be easily distinguished from paraclones. (*b*) RTA in several DU145 holoclones measured using the TRAP assay. (*c*) The telomere lengths of various isolated DU145 holoclones compared with the telomeres of the total population of cells using TRF.

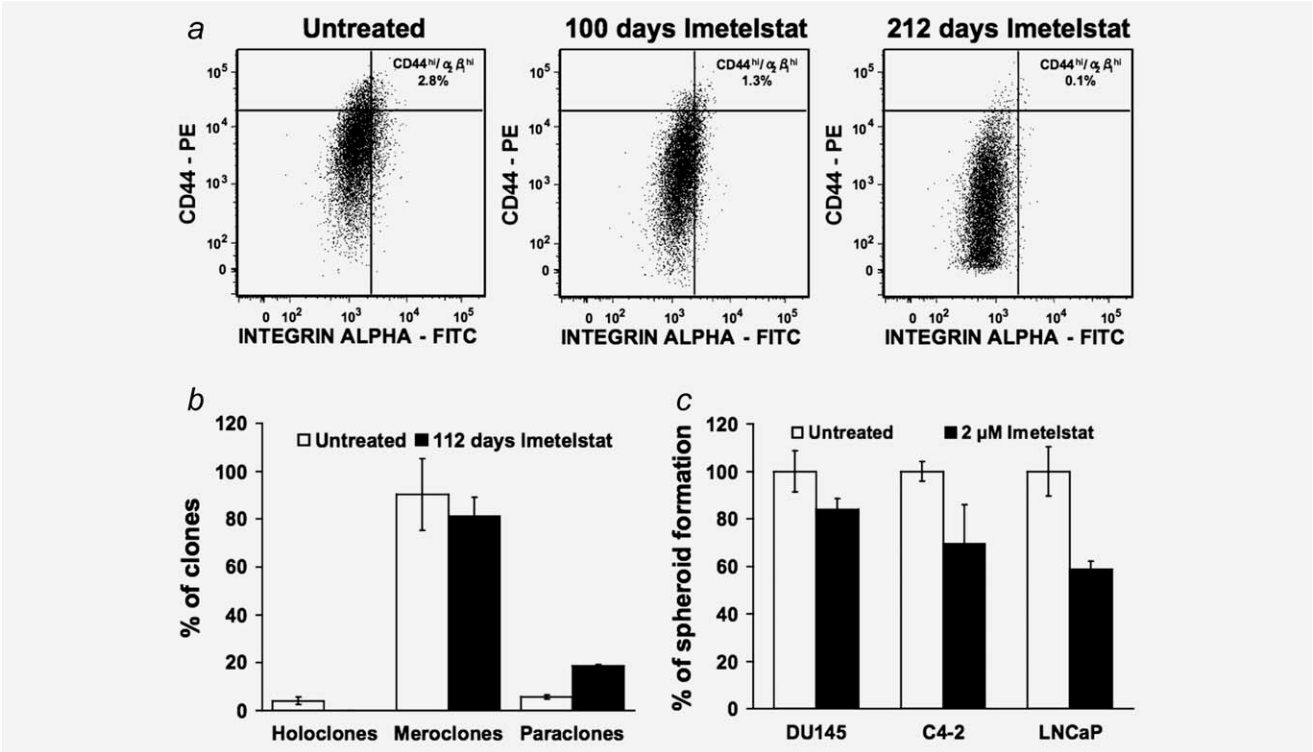


**Figure 6.** Sustained telomerase inhibition by imetelstat in DU145 cells might lead to a decrease in the number of TICs. (*a*) FACS analysis of imetelstat-treated DU145 cells over long periods of time indicates a decrease in the $CD44^{hi}$/integrin $\alpha_2\beta_1{}^{hi}$ tumor-initiating population of cells. (*b*) After prolonged treatment with imetelstat, the capacity of DU145 cells to generate holoclones was completely abolished while the number of paraclones increased. (*c*) Prostate cancer cell lines treated with 2 μM imetelstat (56 days for LNCaP and C4-2; 98 days for DU145) show decreased capacity of self-renewal as indicated by the clonogenic spheroid formation assay.

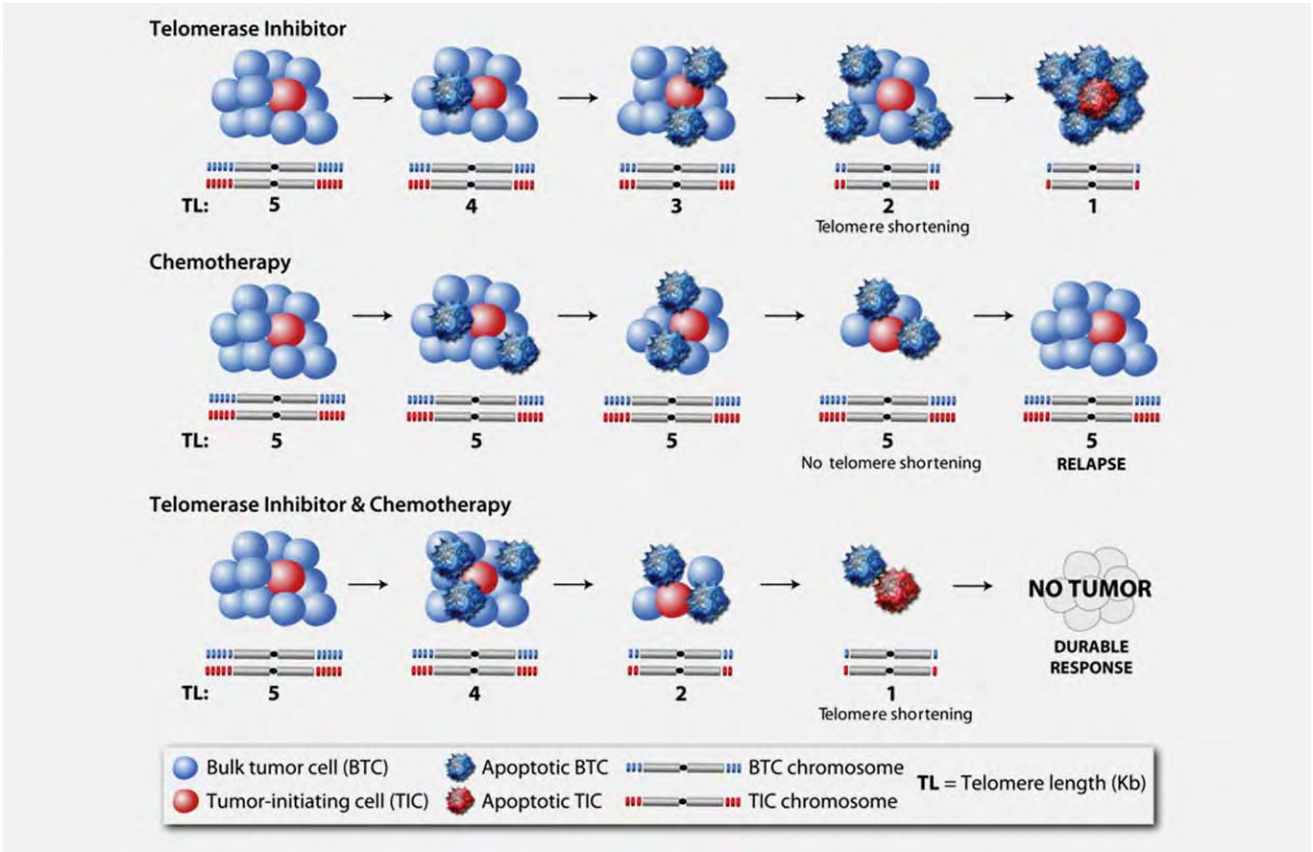


**Figure 7.** Telomerase inhibition in combination with standard chemotherapies may provide a durable response in cancer patients. Telomerase inhibition as a single agent may require a sustained period of telomere erosion to achieve tumor shrinkage. Chemotherapy has a rapid effect on the majority of the tumor cells, but may leave the tumor-initiating compartment intact leading to cancer relapse. Combination of telomerase inhibition and conventional chemotherapy may eliminate the tumor-initiating population while maintaining the tumor size at a manageable level, leading to durable responses.



characteristic of tumor cells. The major aim of this research was to investigate the effects of telomerase inhibition on prostate TICs also referred to as cancer stem cells, using the telomerase antagonist imetelstat (GRN163L). The first question we sought to answer was if prostate TICs have telomerase activity. We chose several cell lines that vary in their androgen responsiveness, tumorigenicity, telomerase activity and telomere lengths. We used subpopulations of cells that demonstrated the highest capacity of tumor initiation (TICs) according to several published studies.[12,15,19,21,23] The methods used to isolate prostate TICs employed established surface markers (CD44, integrin $\alpha_2\beta_1$, CD133), the ability to exclude Hoechst 33342 dye, or the capacity to form holoclones with tumor initiation potential. The results of our experiments show that prostate TICs have significant levels of telomerase activity as measured by the TRAP (telomerase activity) assay. The telomerase activity of TICs was similar not only to the main population of cells, but also to the TIC-negative fractions (Supporting Information Fig. 1). In this study, we chose to focus on the comparison between the TIC fractions and the main population of cells, because the populations of cells that are negative for the cancer stem cell markers used in this study are not capable of tumor initiation and therefore are of little therapeutic significance. This is important, because the reactivation of this enzyme in the TIC compartment is still a topic of debate. One hypothesis, based on normal stem cell biology, was that TICs may be more quiescent than the majority of rapidly dividing cells in the tumor mass. We previously reported that human tumor cells made quiescent by removal of growth factors downregulated telomerase activity.[53] If the prediction that TICs are quiescent was correct, then telomerase activity in TICs would be absent or present at very low levels, similar to normal stem cells. However, based on the available data we cannot completely exclude the possibility that TICs possess significant levels of telomerase activity, despite their quiescent status, and this hypothesis raises interesting questions regarding the role of telomerase in these cells. The present study clearly documents that TICs have significant levels of telomerase activity, similar to the main tumor cell population. This supports the hypothesis that TICs are actively proliferating cells with typical cancer telomerase activity. Importantly, because TICs have similar levels of telomerase to the main population of cells, we hypothesized that treatment with the telomerase

inhibitor imetelstat would have similar effects in this compartment. The experimental data in the present study show that imetelstat treatment efficiently inhibits telomerase activity in all the prostate TICs populations analyzed.

Because there is not always a direct correlation between telomerase activity and average telomere lengths in various cultured prostate cancer cells, it was important to determine the average telomere length of TICs. We found that the telomeres of these cells were approximately the same length or shorter than the average telomere size found in the main population; therefore, we assumed that telomerase inhibitors currently being tested in clinical trials will induce telomere attrition in the rare populations of TICs with equal efficiency as the bulk tumor cells. We were able to show that the telomerase inhibition by imetelstat induced telomere shortening in the TIC compartment and postulate that prolonged telomerase inhibition will lead to apoptosis and cell death of TICs, similar to the main population of cells.

Investigating the telomere length of prostate TICs was important, since it was theoretically possible for these cells to have longer telomeres, similar to normal stem cells. The reduced telomere length in the tumor-initiating compartment may also shed some light on the process of malignant transformation in prostate. Telomere shortening can be detected as early as prostatic intraepithelial neoplasia (PIN) and is restricted to the luminal compartment.[54,55] This suggests that the TICs originate from a subset of transient amplifying cells which under chronic inflammation pressure and genomic instability caused by short telomeres reactivate telomerase and after additional mutations lead to prostate cancer.

Another important observation we made relates to the effects of prolonged telomerase inhibition on the fraction of TICs present in the population. The experimental data shows that long-term treatment with imetelstat leads to a decrease in the number of DU145 $CD44^{hi}$/integrin $\alpha_2\beta_1{}^{hi}$ cells present in the total population of cells due to a reduction in the fluorescence of the whole population. Interesting, the CD133+ cells did not show the same trend (data not shown). Prolonged treatment with imetelstat also correlated with a decreased capacity of DU145 cells to form holoclones. Because one of the main characteristics of TICs is their capacity of self-renewal, it was important to investigate the impact of long-term treatment with imetelstat on the capacity of cells to form spheroids when plated at clonal density in attachment-independent conditions, which was used as a measure of self-renewal capacity for prostate cancer cells.[56] When we performed clonogenic spheroid formation assays on long-term imetelstat-treated LNCaP, C4-2 and DU145 cells, telomere shortening positively correlated with a decrease in the sphere-forming ability of these cells, indicating a decreased capacity of self-renewal. This is important, because it was well documented by our group and others[57] that telomere shortening is associated with a decreased tumor formation ability in immunocompromised mice.

These experiments support the hypothesis that long-term telomerase inhibition by imetelstat coupled with telomere shortening may lead to the elimination of certain populations of prostate TICs. Whether this is a direct result of the elimination of TICs from the population remains unclear and future experiments will be aimed at answering this important question.

One of the ongoing concerns about telomerase inhibition therapy is related to the effects of long-term telomerase inhibition on normal cells. However, normal prostate cells lack telomerase activity and have longer telomere lengths compared to cancer cells.[31,58] Moreover, we have shown that treatment with imetelstat has no effect on the proliferation of normal cells.[46] Normal stem cells are known to be relatively quiescent and have low or no telomerase activity except when dividing. Most importantly, normal stem cells possess relatively long telomeres,[59] and we predict that the effect of imetelstat on these normal stem cells would be less toxic in comparison to the shorter telomere length of TICs. Thus, there may be an optimal therapeutic window that would lead to cancer cell death without irreversibly affecting the normal cells.

Telomerase inhibition as single agent therapy is believed to be most effective only after critical telomere erosion occurred, and this may require relatively long periods of treatment (depending on the initial average telomere length of the tumor) to achieve a reduction in tumor mass. In contrast, conventional therapies (such as surgery, radiation and chemotherapy) lead to a dramatic reduction in tumor burden relatively quickly, but do not lead to durable responses in advanced stage cancers. This may be due to the inherent resistance of TICs to conventional therapeutic agents, behavior strongly documented in glioblastoma.[60–62] We believe that an ideal prostate cancer therapy should combine conventional therapeutic approaches with telomerase inhibitors, such as imetelstat. While conventional approaches will initially target the bulk tumor, after a certain interval of time imetelstat-mediated telomerase inhibition will shorten the telomeres in the tumor-initiating compartment to a critical level, inducing cell apoptosis and death in this small fraction of cells. This therapy approach could potentially lead to durable responses (Fig. 6).

In summary, this preclinical study shows that telomerase inhibition has a great potential for the treatment of prostate cancer and may be able to target the TICs that contribute to relapse and metastasis.



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The Prostate 9999:1–11 (2010)

# Evidence of Epithelial to Mesenchymal Transition Associated With Increased Tumorigenic Potential in an Immortalized Normal Prostate Epithelial Cell Line[Q1]

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**BACKGROUND.** The majority of established human prostate cancer cell lines are derived from metastatic lesions and are already tumorigenic in vivo, therefore immortalized normal prostate cell lines may provide a more relevant model to unveil the mechanisms associated with cancer progression and metastasis.
**METHODS.** PZ-HPV-7, an immortalized human prostate epithelial cell line was used to generate xenograft tumors in mice. A subline designated HPV-PZ-7T was subsequently derived from the subrenal capsule xenograft of a nude mouse. These cells were further characterized using karyotyping, immunofluorescence, qRT-PCR, Western blotting, and three-dimensional cultures in Matrigel.
**RESULTS.** The PZ-HPV-7 cell line possesses a typical epithelial morphology, expresses basal cell markers, and is capable of forming web-like structures with evidence of budding on Matrigel. PZ-HPV-7 is non-tumorigenic in immunocompromised mice by either subcutaneous injection or subrenal grafting. In contrast, the PZ-HPV-7T cells, derived from a xenograft tumor induced by co-inoculation with matrigel using subrenal grafting, possess a mesenchymal phenotype as well as luminal cell markers and are highly tumorigenic and metastatic in nude mice. Functionally and biochemically, the PZ-HPV-7T subline appears to have undergone an epithelial-to-mesenchymal transition (EMT) from the parental PZ-HPV-7 line.
**CONCLUSION.** We have developed a novel EMT model using an immortalized normal prostate epithelial cell line and generated a new prostate cancer cell line, PZ-HPV-7T, which may represent an excellent system to study mechanisms associated with prostate cancer progression and metastasis. *Prostate 9999: 1–11, 2010.* © 2010 Wiley-Liss, Inc.

*KEY WORDS:* **prostate cancer; metastasis; xenografts**

## INTRODUCTION

Prostate cancer is a common malignancy found in humans and one of the most lethal cancer types in men [1]. The genetic mouse models which are commonly used to study the early stage of prostate carcinogenesis may not fully represent the human disease. In addition, the majority of human prostate cancer cell lines derived from metastatic sites were established and maintained using serum-supplemented medium [2,3], which is known to maintain a differentiated phenotype. For example, the LNCaP and LAPC4 cell lines were derived from the lymph node of a patient with metastatic prostate cancer, while the DU-145 and PC3 cell lines were derived from brain and bone metastasis

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respectively [4]. These cell lines are tumorigenic in immunocompromised mice and may already represent the late stage of prostate carcinogenesis. Thus, there is a critical need for new human prostate carcinogenesis models, particularly those derived from normal prostate epithelium, in order to provide a better understanding of early events in prostate cancer development.

Epithelial-to-mesenchymal transition (EMT) is a highly conserved cellular process that allows the polarized and generally immotile epithelial cells to convert to motile mesenchymal cells [5]. This important process was initially recognized during several critical stages of embryonic development and has recently been implicated in promoting cancer invasion and metastasis [6]. A decrease in the expression of E-cadherin and an increase in the expression of vimentin are two currently accepted biochemical characteristics associated with EMT [7].

In prostate cancer, decreased E-cadherin expression has been correlated with various indices of prostate cancer progression, including grade of the cancer, local invasiveness, dissemination into blood, and tumor relapse after radiotherapy [8]. The critical role of EMT in prostate cancer progression has not been completely understood since most studies were performed using established human prostate cancer cell lines.

In this report, the PZ-HPV-7 cell line derived from histological normal prostate epithelium immortalized by Human Papilloma Virus Type 18 (HPV-18) DNA [9] was employed to establish a malignant model of EMT. PZ-HPV-7 cells are generally considered as non-tumorigenic in subcutaneous xenograft animal models [10]. Nevertheless, we have established a new PZ-HPV-7T line from the parental PZ-HPV-7 cells which is highly tumorigenic in nude mice and has increased expression of EMT markers, suggesting the progression to a more aggressive phenotype. In conclusion, we believe that this new prostate cancer line provides a useful model for studying EMT and prostate carcinogenesis.

## MATERIALS AND METHODS

### Cell Lines and Culture Conditions

The PZ-HPV-7 cell line was a kind gift from Dr. Donna Peehl (Stanford University School of Medicine, USA). All the other cell lines were purchased from American Type Culture Collection (Manassas, VA). The PZ-HPV-7 cells were maintained in a chemical defined prostate epithelial cell growth media (PrEGM, Lonza, Walkersville MD). All the other cells lines were cultured in T-medium (Invitrogen, Carlsbad CA) with 5% FBS (unless otherwise specified). Spheroids were generated by mixing 1:1 cells suspension in culture media (60,000/ml) with Matrigel (BD Biosciences, San Jose CA) on ice. The cells/Matrigel mixture was immediately placed in six well plates (Fisher Scientific, Pittsburgh, PA) and the culture medium was refreshed every other day. In order to generate web-like structures with buds, the Matrigel was placed in the dishes, allowed to solidify at 37°C, and then the cells were plated on top of the Matrigel in culture media.

### Renal Capsule Grafting

The detailed procedure of renal capsule grafting is described and illustrated at the website (http://mammary.nih.gov/tools/mousework/Cunha001/index.html). Briefly, male athymic nu/nu homozygous nude mice (6–8 weeks old) were anesthetized, and the kidney was exposed through a dorsal incision. An incision was made in the capsule, and cell pellet with 2 mm × 2 mm × 2 mm Gelfoam (Pfizer, New York, NY) was placed underneath the capsule. About 2 million of cells were placed per kidney at the left side. After grafting, the kidney was placed back into its position, the muscle layer was closed with 6-0 absorbable sutures, and the skin was stapled. After 10 weeks, mice were sacrificed and the grafts were removed for analysis.

### Cytogenetic Analysis

Metaphase chromosomes were obtained from in situ cultures of PZ-HPV-7 and PZ-HPV-7T cells. Giemsa trypsin G-banding (GTG) was performed according to the standard procedure. At least 20 metaphases cells per cell line were analyzed.

### Estimation of Telomerase Activity and Telomere Length

Telomerase activity was measured using the Telomeric Repeat Amplification Protocol (TRAP) described previously [11]. The telomerase activity is visualized as a 6-bp ladder after running the PCR products on 10% non-denaturing acrylamide gel and using a Typhoon Trio Variable Mode Imager (Amersham Biosciences, Piscataway, NJ). A 36-bp internal amplification control was used to monitor the efficiency of the PCR reaction. Telomere lengths were measured using the Terminal Restriction Fragment (TRF) assay. Total DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen Sciences, MD). One microgram of total DNA was incubated with a mixture of six enzymes (*Alu*I, *Cfo*I, *Hae*III, *Hin*fI, *Msp*I, and *Rsa*I) for 6 hr at 37°C and then separated on a 0.7% agarose gel overnight at 70 V. The gel was denatured in a solution of 1.5 M NaCl and 0.5 M Tris–HCl pH 8.0 for 20 min, rinsed in distilled water and dried for 2–3 hr at 55°C. The gel was then

incubated in neutralizing solution (1.5 M NaCl, 0.5 M Tris–HCl pH 8.0) for 15 min and hybridized with a $^{32}$P-labeled telomeric probe overnight at 42°C. The gel was washed in 2× SSC for 15 min, then washed twice in 0.1× SCC plus 0.1% SDS for 10 min and rinsed again in 2× SCC. The gel was exposed to a phosphor screen overnight and analyzed using a Typhoon Trio Variable Mode Imager (Amersham Biosciences).

### Immunofluorescent Staining

The spheroids were fixed in 10% neutral buffered formalin, permeabilized for 20 min with 0.1% Triton X-100 in 1× TBS, then blocked with 5% nonfat dry milk in 1× TBS-T for 1 hr at room temperature. Incubation with the primary antibodies such as monoclonal mouse 34βE12, polyclonal prostate-specific antigen (PSA) (Dako, Glostrup, Denmark); rabbit androgen receptor (AR) and mouse cytokeratin 18 (CK18) (Sigma, St. Louis, MO) was performed overnight at 4°C on a tilting table. After three washes in TBS-T, the spheroids were incubated with the secondary antibodies (AlexaFluor 568 and AlexaFluor 488, Invitrogen) for 1 hr at room temperature. After three more washes with TBST, the spheroids were mounted in Vectashield with DAPI (Vector Labs, Burlingame, CA) and examined using a Zeiss Axiovert 200 inverted microscope.

### Wound Healing and Matrigel Invasion Assays

The wound-healing assay was performed by growing cells to confluence then creating a scratch on the monolayer using a sterile pipette tip. The cell migration was monitored under microscope until the wound closed. For the invasion assays, $5 \times 10^4$ cells/ml were plated in a BD BioCoat Matrigel Invasion Chamber (BD Biosciences) in serum-free media. Chemoattractants (5% FBS or growth factors) were placed in the lower chamber and the plates were incubated for 22 hr in a humidified tissue culture incubator at 37°C, 5% $CO_2$ atmosphere. After the removal of non-invading cells, the invading cells were fixed in 100% methanol and stained with 1% toluidine blue (in 1% borax solution).

### qRT-PCR Analysis

The total cellular RNA was extracted with RNeasy mini kit (Qiagen, Valencia, CA) treated with RNase-free DNase I (Qiagen). A total of 1 μg RNA was subjected to a cDNA synthesis kit (Bio-Rad, Hercules, CA). One-tenth of the cDNA was subjected to a 25-μl PCR reaction carried out in an iCycler thermal cycler (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad) using PCR primers (sequences available on request) and 18S RNA primer sets (0.6 ng/μl). All experiments were repeated at least twice in duplicates. The relative level of mRNA from each sample was determined by normalizing with 18S cDNA.

### Western Blot Analysis

Total cellular proteins were extracted with a protein lysis buffer [50 mM HEPES (pH 7.5), 150 nM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$] containing protease inhibitors. The protein samples were separated by electrophoresis on a polyacrylamide gel (NuPAGE 10% bis-Tris gel, Invitrogen) and transferred to nitrocellulose membranes. The membranes were incubated with the primary antibodies for vimentin, fibronectin, actin (Sigma), N-cadherin, E-cadherin, HIF-1α (BD Biosciences), DAB2IP (Zymed, San Francisco, CA), and ERβ (Santa Cruz Biotechnology, Santa Cruz, CA). The VEGF-A antibody was kindly provided by Dr. Rolf Brekken (University of Texas Southwestern Medical Center, Dallas, TX). After incubation with the secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA), the membranes were developed using the Amersham ECL Plus (GE Healthcare, Piscataway, NJ) or SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL).

## RESULTS

### Tumorigenicity of PZ-HPV-7 Cells in Immunocompromised Mice

Consistent with previous reports [9], the PZ-HPV-7 cells appeared to be non-tumorigenic in nude mice (Table I) when implanted by subcutaneous injection (0/2) or subrenal grafting (0/8). However, when PZ-HPV-7 cells (68th passage) were cultured as spheroids in Matrigel then implanted under the renal capsule of nude mice, one mouse (1/5) developed a palpable xenograft tumor (Fig. 1A and Table I). The tumor was excised, placed back into cell culture and the cell line generated from this xenograft explant was designated PZ-HPV-7T. The PZ-HPV-7T cells appeared to be more tumorigenic and metastatic than the parental PZ-HPV-7 cells (Table I). Mice inoculated with PZ-HPV-7T cells under the renal capsule exhibited multiple sites of metastases such as lymph nodes and lungs (Fig. 1A). In addition to its capacity to form tumors in the subrenal capsule of nude mice, PZ-HPV-7T cells were able to generate subcutaneous tumors in NOD/SCID mice (Table I). In two-dimensional cultures on plastic dishes, PZ-HPV-7 cells have typical epithelial morphology, but the PZ-HPV-7T cells possess a more mesenchymal-like appearance (Fig. 1B).

### PZ-HPV-7 and PZ-HPV-7T Cells Karyotypes

Representative karyotypes for PZ-HPV-7 and PZ-HPV-7T cells are shown in Figure 2A,B. PZ-HPV-7 cells

**TABLE I. Xenograft Tumor Formation in Immunocompromised Mice**

| Cell type/growth conditions | Inoculation site | No. of mice | % Tumors |
|---|---|---|---|
| PZ-HPV-7/monolayer | Subrenal | 8 | 0% (0/8) |
| PZ-HPV-7/spheroids | Subrenal | 5 | 20% (1/5) |
| PZ-HPV-7/monolayer | Subcutaneous | 2 | 0% (0/2) |
| PZ-HPV-7T/monolayer | Subrenal | 4 | 100% (4/4) |
| PZ-HPV-7T/spheroids | Subrenal | 4 | 75% (3/4) |
| PZ-HPV-7T/monolayer | Subcutaneous | 2 | 100% (2/2) |

are hypertriploid with the chromosome number ranging from 78 to 85, with deletion of chromosomes 3p, 15, 21, and 22, as well as gain of chromosomes 3q, 5, 5p, 7, 7p, 9q, 11, 16p, 17, 18, 19, 20, and 3–7 marker chromosomes (Fig. 2A). PZ-HPV-7T cells are hyperdiploid with the chromosome number ranging from 47 to 57. Deletion of chromosomes 4, 7q, 16q, 18, and 20 as well as gain of chromosomes 1, 5p, 6p, 8, 9q, 10q, 11q, and 17 can be observed (Fig. 2B). Structural anomalies including an additional isochromosome 5p and a 7q deletion from 7q11.2 to the 7qter region were present in both cells, supporting the common origin of these two cell lines.

### Determination of Telomerase Activity and Telomeres Length

Because telomerase activation is one of the hallmarks of malignant transformation, we examined the telomerase activity in PZ-HPV-7 and PZ-HPV-7T cells. The TRAP assay shows significant levels of telomerase activity in both cell types (Fig. 3A). In contrast, normal prostate epithelial cells lack telomerase activity (data not shown). Telomerase activation is believed to be a result of critically short telomeres during prostate carcinogenesis; therefore we analyzed the telomere lengths of PZ-HPV-7 and PZ-HPV-7T cells. The PZ-HPV-7 cells have relatively short telomeres, with an average of 4.2 kb (Fig. 3B). PZ-HPV-7T cells have slightly longer telomeres, in the range of 7 kb (Fig. 3B).

### Expression of Basal and Luminal Makers in PZ-HPV-7 and PZ-HPV-7T Cells

In order to further investigate characteristics of PZ-HPV-7T cells, we performed an immunofluorescent analysis using spheroids growing in Matrigel. PZ-HPV-7 cells possess a basal phenotype as indicated by

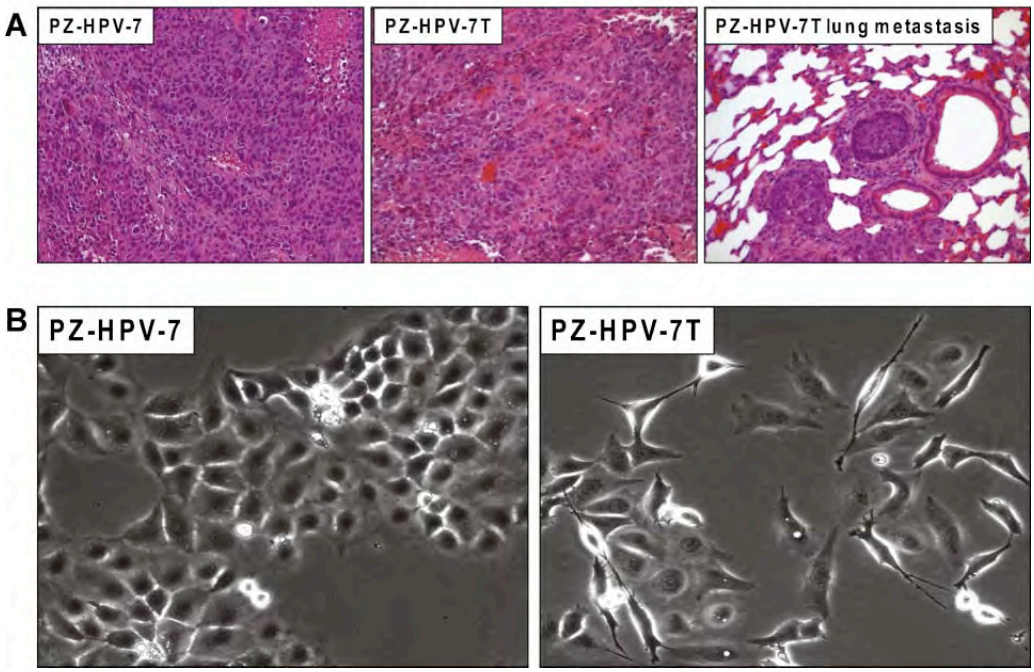


**Fig. 1.** The morphology of PZ-HPV-7 and PZ-HPV-7T cells and the histology of tumors in mouse xenografts. **A**: H&E staining of the mouse subrenal xenograft tumors generated from PZ-HPV-7 and PZ-HPV-7T cells. The PZ-HPV-7T xenografts show evidence of lung metastasis. **B**: Morphology of the PZ-HPV-7 and PZ-HPV-7T cells growing on a on a plastic substrate.

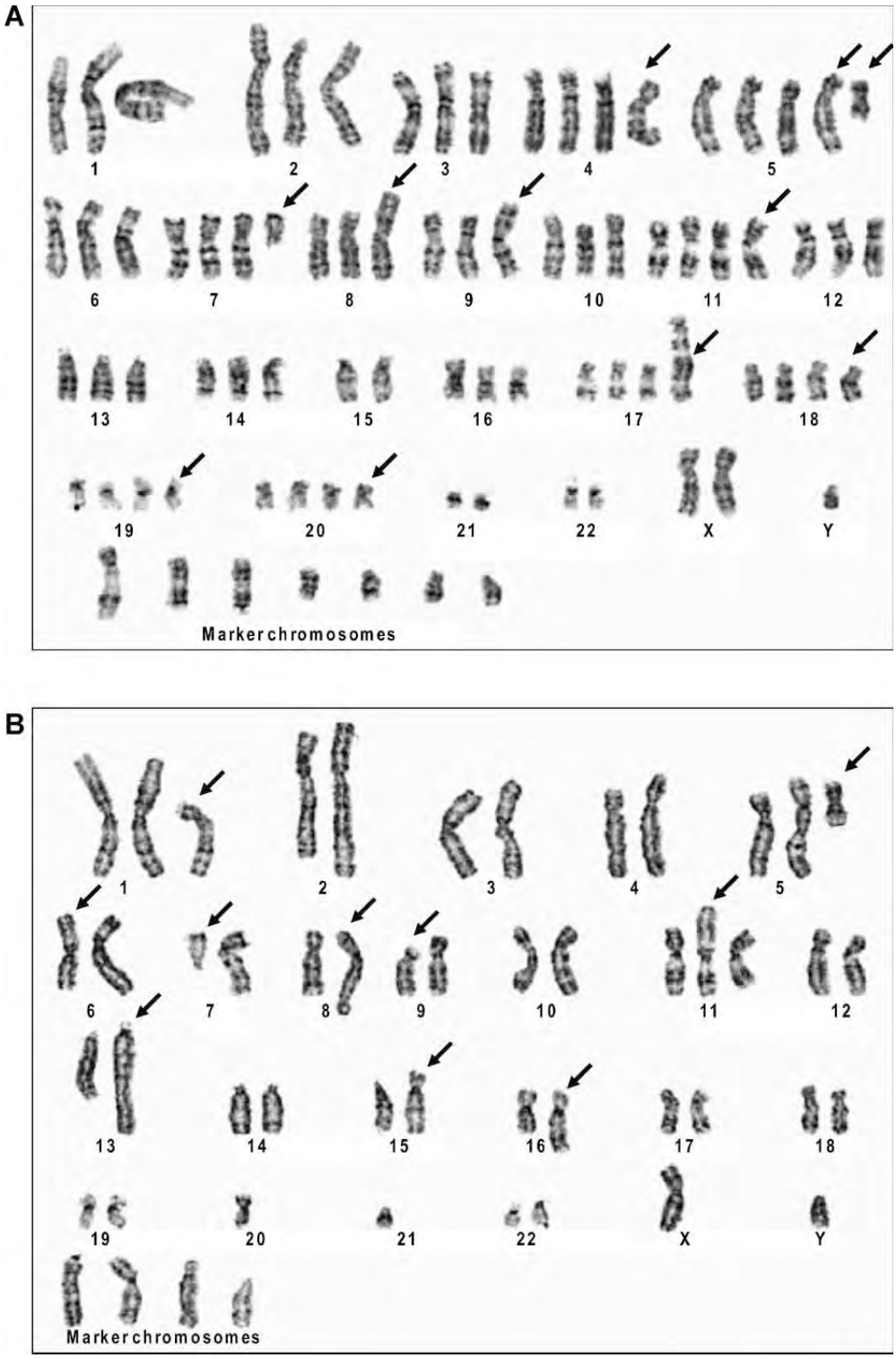


**Fig. 2.** Representative karyotypes for PZ-HPV-7 and PZ-HPV-7cells. **A**: Late passage PZ-HPV-7 cells were hypertriploid with the following karyotype: 82 <3n>, XXY, +4, +5, +i(5)(p10), +del(7)(q11.2), der(8)t(8;10)(p11.2;q11.2), i(9)(q10), +11, −15, +der(17)t(5;17)(q13;q25), +18, +19, +20, −21, −22, +7mar; (**B**) PZ-HPV-7T cells presented a hyperdiploid karyotype with: 51 <2n>, XY, +t(1;11)(p22;p11.2), +i(5)(p10), der(6)add(6)(p23)del(6)(q23), del(7)(q11.2), add(8)(q24), del(9)(p13), add(13)(q34), add(15)(p12), add(16)(q22), −20, −21, +4mar. The chromosomal aberrations are indicated by arrows.

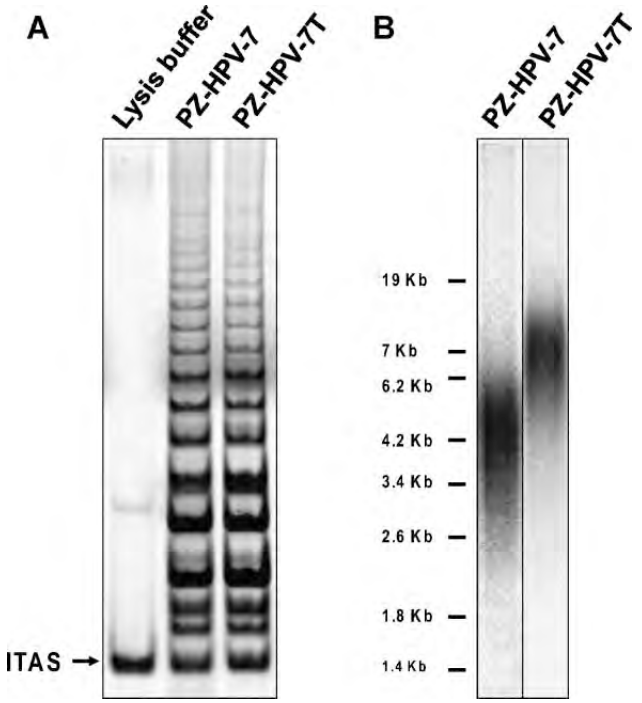


**Fig. 3.** Telomerase activity and telomere length in PZ-HPV-7 and PZ-HPV-7 cells. **A**: Telomere repeat amplification protocol (TRAP) on cell lysates from PZ-HPV-7 and PZ-HPV-7T cells. The internal amplification standard was indicated by an arrow. **B**: Terminal restriction fragment (TRF) assay on telomere DNA extracted from the PZ-HPV-7 and PZ-HPV-7T cell lines. Molecular size was indicated on the left.

the presence of high molecular weight cytokeratins detected by the 34βE12 antibody but not CK18 (an established marker for prostate luminal cells) (Fig. 4). In contrast, the PZ-HPV-7T cells were CK18-positive, similar to the majority of prostate cancer expressing luminal markers [4]. However, both PZ-HPV-7 and PZ-HPV-7T cells are AR- and PSA-negative (Fig. 4) at protein level. These results were confirmed by Western blots (data not shown).

### Increased Motility and Invasiveness in PZ-HPV-7T Cells

The xenograft experiments clearly illustrated that PZ-HPV-7 cells are less tumorigenic than the PZ-HPV-7T cells. We sought to determine whether the high tumorigenicity of PZ-HPV-7T cells was associated with increased motility and invasiveness in vitro. The PZ-HPV-7T cells have significantly higher motility than the PZ-HPV-7 cells as indicated by the wound-healing assay (Fig. 5A). After 30 hr, the wound in the cells monolayer was completely closed for both the positive control PC3 cells and the PZ-HPV-7T cells. In contrast, PZ-HPV-7 cells achieved only half of the complete wound healing potential, indicating much lower cell motility. Next, we analyzed the capacity of PZ-HPV-7T cells to invade through Matrigel using a modified Boyden chamber assay. PZ-HPV-7T cells are highly invasive, as indicated by the high number of cells present on the membranes (Fig. 5B). In fact, it appears that PZ-HPV-7T cells have similar invasion potential to the PC3 cell line, which is known to be highly tumorigenic and invasive. Not surprising, PZ-HPV-7

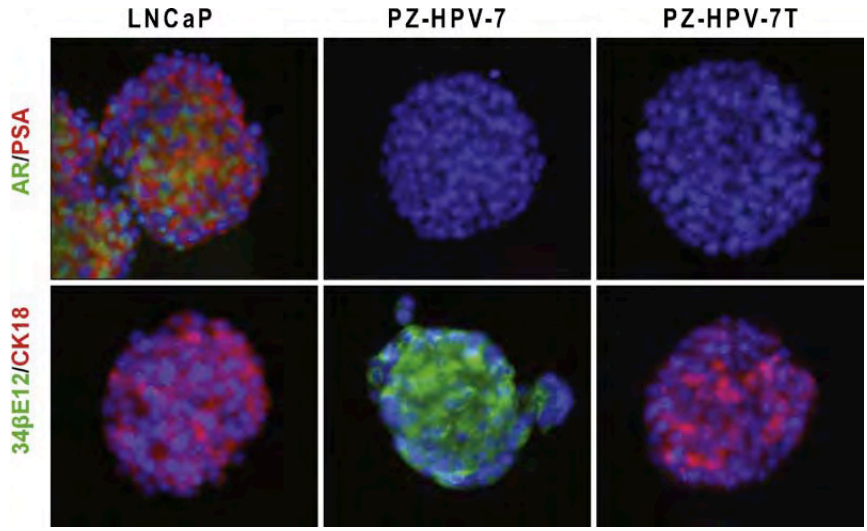


**Fig. 4.** Immunofluorescent staining of spheroids growing in Matrigel. The PZ-HPV-7 and PZ-HPV-7T cells were negative for AR and PSA (**upper panels**). The PZ-HPV-7 cells expressed high molecular weight cytokeratins (using the 34βEI2 antibody) while the PZ-HPV-7T cells expressed CK18 (**lower panels**). LNCaP cells were used as control. AR and 34βEI2 were visualized with green AlexaFluor488 while PSA and CK18 with red AlexaFluor568. The cells were counterstained with DAPI (blue).

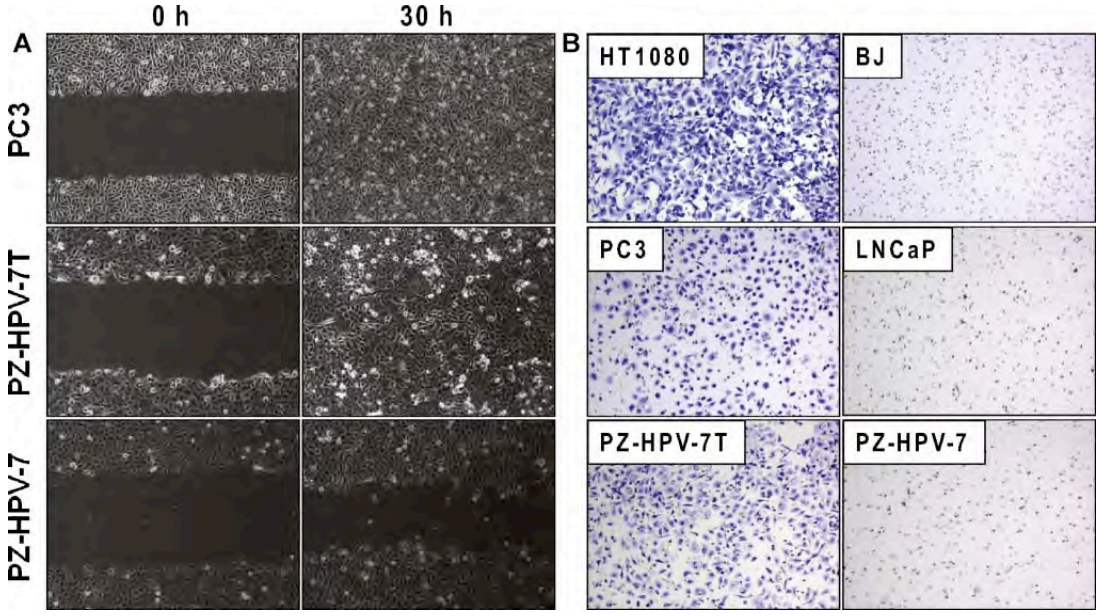


**Fig. 5.** Determination of motility and invasiveness in PZ-HPV-7 and PZ-HPV-7T cells. **A**: Increased motility for PZ-HPV-7T cells 30 hr after scratching. The PC3 cells were used as positive control. **B**: Increased invasive potential of PZ-HPV-7T cells through Matrigel. Only the cells that could invade through the BD Biocoat inserts in response to chemoattractants were stained with toluidine blue. HTI080 and PC3 cells were used as positive controls, while BJ and LNCaP cells were used as negative controls.

cells have very low invasive potential, similar to the LNCaP cells used in the assay (Fig. 5B).

### Formation of Web-Like Structures in PZ-HPV-7 Cells Cultured on Matrigel

The capacity of prostate epithelial cells to form webs, ducts and acinar-like structures when cultured on basement membrane matrices was associated with a less tumorigenic phenotype, while more tumorigenic cells produced unorganized cell aggregations [12,13]. We set out to test whether PZ-HPV-7 cells are capable of forming organized structures when cultured on Matrigel and the data demonstrated that these cells generated web-like structures with evidence of budding (Fig. 6). In contrast, PZ-HPV-7T cells lost their ability to form these structures and grew as multicellular spheroids on the Matrigel surface, indicating the transition to a more aggressive phenotype.

### Significant Changes of EMT Markers in PZ-HPV-7T Cells

Because EMT has been associated with increased invasive and metastatic potential in different cancer types [5,6], we examined whether the PZ-HPV-7T cells underwent such transition using several well-characterized markers. To rule out possible artifacts due to different growth media, we have grown PZ-HPV-7T in either serum-free PrEGM (P-M) or T-medium (T-M) containing 5% serum. The expression levels of ZEB1 and ZEB2 mRNA were significantly elevated in PZ-HPV-7T cells, regardless of the culture conditions (Fig. 7A). The expression of Snail and FOXC2 mRNA, two other key players in the EMT process, were also significantly elevated in PZ-HPV-7T cells in both media. Similarly, vimentin, N-cadherin and fibronectin, bona fide mesenchymal markers, were elevated in the PZ-HPV-7T cells (Fig. 7B) in both culture media. In contrast, E-cadherin, an epithelial marker, decreased significantly in PZ-HPV-7T cells compared with PZ-HPV-7 cells (Fig. 7B). Interestingly, decreased expression of DAB2IP protein, a critical factor in preventing EMT [14], was also detected in PZ-HPV-7T cells but not the PZ-HPV-7 cells. A recent study demonstrated that the presence of ERβ in prostate cancer cells is capable to repress EMT by destabilizing both HIF-1α and VEGF-A [15]. Thus, we profiled the expression levels of ERβ, as well as HIF-1α and VEGF-A in these cells. As indicated in Figure 7B, elevated levels of ERβ and VEGF-A proteins were detected in PZ-HPV-7T cells, but this increase was not seen for HIF-1α, which was almost undetectable in both cells. Taken together, these results indicate that the signal pathway(s) leading to EMT in PZ-HPV-7T cells is independent from the ERβ-mediated pathway.

## DISCUSSION

Unlike human keratinocytes [16], previous studies indicated that introduction of HPV-18 is not sufficient

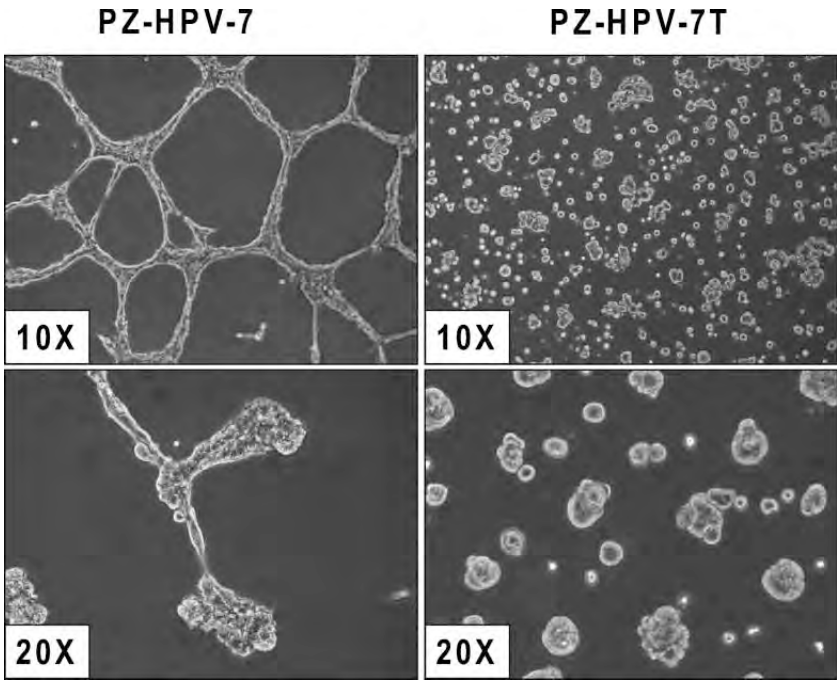


**Fig. 6.** Formation of web-like structures by PZ-HPV-7 cells cultured on Matrigel. Cells were plated on top of the Matrigel and photographed 24 hr later using a phase-contrast microscope. PZ-HPV-7 cells exhibited budding and acini-like structures. PZ-HPV-7T cells formed multicellular spheroids, indicating a tumorigenic phenotype.

to induce malignant transformation in normal prostate cells [17], which is consistent with the reported PZ-HPV-7 phenotype [9]. Nevertheless, in organotypic cultures, some HPV-immortalized cells are known to exhibit a pre-malignant phenotype [18]. While early passages of PZ-HPV-7 cells have a diploid karyotype, later passages give rise to cytogenetics aberrations, also consistent to a pre-malignant phenotype [10]. Based on

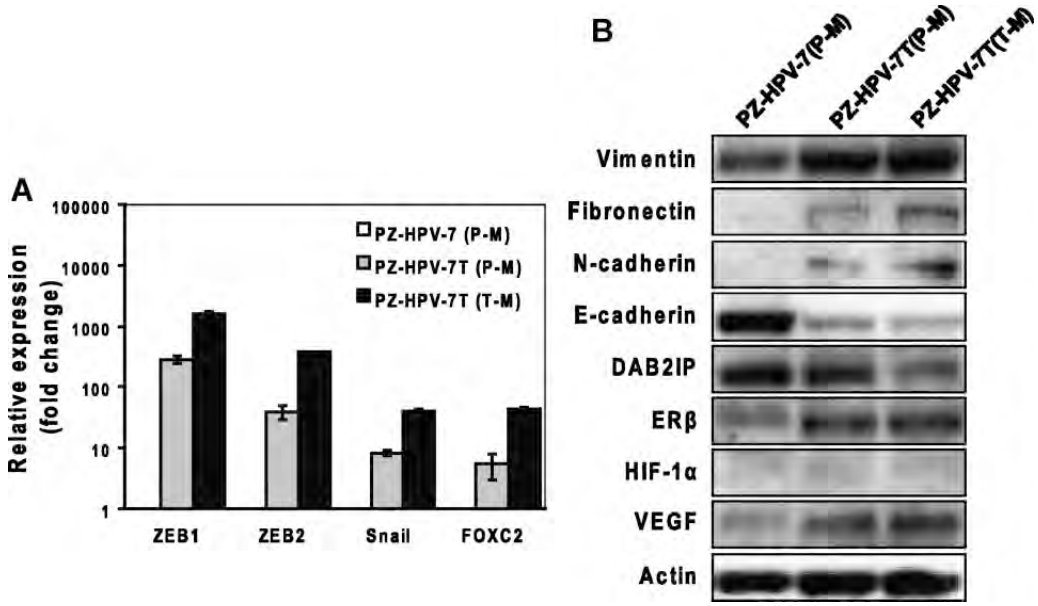


**Fig. 7.** Characterization of EMT changes in PZ-HPV-7 and PZ-HPV-7T cells. **A**: Steady-state mRNA levels of ZEBI, ZEB2, Snail, and FOXC2 in PZ-HPV-7 and PZ-HPV-7T cells were determined by qRT-PCR. Asterisk represented statistical significant between PZ-HPV-7Tand PZ-HPV-7 ($P < 0.05$). **B**: Protein levels of vimentin, fibronectin, N-cadherin, E-cadherin, DAB2IP, ERβ, HIF-1α, and VEGF-A in PZ-HPV-7 and PZ-HPV-7T were analyzed by Western blots. Actin was used as loading control. The cells were grown in Matrigel as spheroids in two different media (P-M, PrEGM medium; T-M, T-medium).

these observations we hypothesized that PZ-HPV-7 cells cultured under organotypic conditions will give rise to tumors in immunocompromised mice. Indeed, PZ-HPV-7 spheroids generated in Matrigel were able to generate tumors in the subrenal capsule of immunocompromised mice after 8 weeks post-inoculation and the tumorigenic and malignant line derived from this tumor was designated PZ-HPV-7T. Certainly, larger animal cohorts will be required to confirm whether spheroids (vs. monolayer) culture conditions are a critical contributor in the xenograft tumor formation, since only small number of animals ($n = 15$) were used in this study.

The PZ-HPV-7 cells plated on Matrigel formed web-like structures, as opposed to the PZ-HPV-7T cells which formed disorganized cell aggregates. This is consistent with previous reports indicating that this attribute is a characteristic of malignant prostate cells with a less tumorigenic phenotype [12,13]. While the PZ-HPV-7 cells form branch-like structures when plated on top of the Matrigel substrate, the cell spheroids generated in the Matrigel do not form acinar structures with lumens (data not shown). Although we cannot dismiss the role of extracellular calcium [19] in acinar formation, the behavior of PZ-HPV-7 on Matrigel is consistent with their pre-malignant phenotype. Noticeably, the PZ-HPV-7 cells were unable to form tumors in immunocompromised mice unless they were maintained as spheroids in Matrigel previous to inoculation. It is known that Matrigel provides a more relevant physiological environment, and this is especially important for studies in which the interaction between the cells and extracellular matrix need to be similar to the native prostate environment [20]. This observation might open new avenues for studying the impact of basement membrane on prostate carcinogenesis, which is used to be difficult to perform in vitro with consistent results.

A previous study have shown that PZ-HPV-7 cells will progress from a normal diploid chromosome number (at early passages) to a hypertetraploid karyotype in late passages [10]. Consistent with this report, we observed hypertriploid chromosomal numbers in the late-passage PZ-HPV-7 cells used in this study. Interesting, the PZ-HPV-7T tumorigenic subline had mostly a diploid chromosome number. One possible explanation is that a small number of cell in the PZ-HPV-7 population maintain a near-diploid karyotype and that these cells are selected by the mouse xenograft environment, leading to the subsequent tumor formation.

Telomerase re-activation is one of the hallmarks of cancer and the majority of prostate cancer cells possess high levels of telomerase activity in contrast to normal prostate or benign prostatic hyperplasia tissues [21,22] which are telomerase negative. Moreover, telomere shortening is one of the earliest events in the prostate malignant transformation and majority of prostate cancer cells possess relatively short telomeres [23]. The pre-neoplastic nature of PZ-HPV-7 cells is therefore supported by the observation that these cells have high levels of telomerase activity and short telomeres. Moreover, PZ-HPV-7T maintains high levels of telomerase activity, which makes it a viable model for screening prostate cancer anti-telomerase agents [24–26].

PZ-HPV-7 cells stained positively with antibodies against high molecular weight cytokeratins (34βE12), suggesting a basal cell origin. PZ-HPV-7 cells grown as a monolayer on plastic are low tumorigenic in immunocompromised mice (0/2 subcutaneous, 0/8 subrenal), but did form a tumor when cultured in Matrigel (1/5 subrenal). Therefore, this model suggests that a very small fraction of the PZ-HPV-7 cell population may include tumor-initiating cells (cancer stem cells). Subsequently, PZ-HPV-7T cells derived from this tumor appear to be highly tumorigenic (~100% tumor take rate in almost all models tested) and express the CK18 marker, often detected in clinical specimens of prostate cancer. Noticeably, the metastatic potential of PZ-HPV-7T cells was confirmed by the in vivo animal model as well as both in vitro motility and invasiveness assays. Considering the higher motility and invasiveness of PZ-HPV-7T cells, these observations suggest the presence of an EMT process that may take place in these cells. EMT is one of the critical events during embryonic development and recent evidence suggest a connection between the EMT, cancer progression and metastasis [5]. In prostate cancer, decreased expression of E-cadherin correlates with cancer progression, invasion and relapse after radiotherapy [7,27] while N-cadherin is up-regulated in cells which adopt a mesenchymal phenotype [28]. The PZ-HPV-7 cells expressed significant levels of E-cadherin when maintained as spheroids in Matrigel. Noticeably, the metastatic PZ-HPV-7T exhibited reduced E-cadherin expression but elevated N-cadherin expression, regardless of different culture media. The reduced level of E-cadherin, along with the loss of prostate acinar architecture is consistent with a de-differentiated phenotype, characteristic to aggressive prostate cancer cells. These data clearly suggest an involvement of EMT. To further strengthen these observations, we analyzed the upstream regulators of E-cadherin expression, namely ZEB1, ZEB2, and Snail. The elevated expression of these genes is consistent with the expected changes observed during EMT. It is well known that these factors serve as suppressors of E-cadherin. In addition to its role as suppressor of E-cadherin, Snail also serves as an activator of vimentin

and fibronectin, which are clearly up-regulated in PZ-HPV-7T cells. In breast cancer it was shown that FOXC2 expression is induced in cells undergoing EMT in response to Snail and Twist transcription factors [29]. We observed the same expression pattern, FOXC2 being significantly up-regulated in PZ-HPV-7T cells. It was previously established that the loss of DAB2IP expression, often detected in prostate cancer specimens, initiates EMT in both prostate cancer cells and prostate epithelia of DAB2IP knockout animals [14]. Interestingly, PZ-HPV-7 cells express detectable levels of DAB2IP, but this is dramatically decreased in the PZ-HPV-7T cells regardless of culture conditions, providing an additional line of evidence to support the presence of EMT in PZ-HPV-7T cells derived from the parental PZ-HPV-7 cells, which is correlated with its tumorigenicity and metastatic potential in vivo.

A recent publication demonstrated the potential role of ERβ in repressing the EMT which is often associated with high-grade prostate cancers [15]. The ERβ mechanism of action in this study was the destabilization of HIF-1α and inhibition of VEGF-A after EMT induction by TGF-β or hypoxia. Indeed, the amount of VEGF-A protein in PZ-HPV-7T cells was higher than in the parental PZ-HPV-7 cells. However, ERβ was not down-regulated in the PZ-HPV-7T cells used in our model. Very likely, the EMT pathways involved in PZ-HPV-7T cells disengage from ERβ-mediated pathway. Thus, PZ-HPV-7T cells may provide a distinct EMT model for studying ERβ-independent pathway(s).

In summary, PZ-HPV-7T cell, generated from an immortalized prostate epithelial cell line, represents a new model for the analysis of tumor initiation, EMT and cancer metastasis. While the two cell lines shares a similar genetic ancestry, they appear to vary significantly in their morphology, invasiveness, and tumorigenicity.

## ACKNOWLEDGMENTS

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